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RESEARCH ARTICLE

Persistent 6-OH-BDE-47 exposure impairs functional neuronal maturation and alters expression of neurodevelopmentally-relevant chromatin remodelers

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Abstract

Polybrominated diphenyl ethers (PBDEs) are a pervasive class of brominated flame retardants that are present in the environment at particularly high levels, especially in the United States. Their environmental stability, propensity for bioaccumulation, and known potential for neurotoxicity has evoked interest regarding their effects on the developing nervous system. Exposure to PBDEs has been strongly associated with neurodevelopmental disorders. However, the details of their mechanistic roles in such disorders are incompletely understood. Here, we report the effects of one of the most prevalent congeners, BDE-47, and its hydroxylated metabolites on the maturation and function of embryonic rat cortical neurons. Prolonged exposure to 6OH-BDE-47 produces the strongest effects amongst the parent BDE-47 congener and its tested hydroxylated metabolites. These effects include: i) disruption of transcriptional responses to neuronal activity, ii) dysregulation of multiple genes associated with neurodevelopmental disorders, and intriguingly, iii) altered expression of several subunits of the developmentally-relevant BAF (Brg1-associated factors) chromatin remodeling complex, including the key subunit BAF170. Taken together, our data indicate that persistent exposure to 6OH-BDE-47 may interfere with neurodevelopmental chromatin remodeling mechanisms and gene transcription programs, which in turn are likely to interfere with downstream processes such as synapse development and overall functional maturity of neurons. Results from this study have identified a novel aspect of 6OH-BDE-47 toxicity and open new avenues to explore the effects of a ubiquitous environmental toxin on epigenetic regulation of neuronal maturation and function.

Key words: neurodevelopment; BDE-47 exposure; Arc; activity-induced transcription; BAF complex

Introduction

Polybrominated diphenyl ethers (PBDEs) are a widely-used class of organohalogenated flame retardants that were popularized—and mandated for use in some cases—in the 1970 s. This has led to their abundance in consumer products including furniture, electronics, and clothing and therefore within human households [1]. Moreover, the environmental stability and strong lipophilicity of these compounds enables their bioaccumulation,

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specifically in lipid rich adipose and brain tissue [2, 3]. These characteristics have raised the issue of potential toxicity of these compounds, leading to the investigation of their effects on human health [4]. Despite known dangers, the usage and manufacturing of specific congeners has only recently become restricted and/or banned in the United States and Europe, and their presence in the environment will persist for decades to come [5]. In addition to parent congeners, toxicity may also arise from metabolic products of PBDEs. In mammals, cytochrome p450 enzymes metabolize PBDEs to form hydroxylated-BDEs (OH-PBDEs) [6], some of which may be over an order of magnitude more toxic than the parent compound (e.g., 6OH-BDE-47) [7–9]. Buildup of such toxic metabolites and their parent PBDEs from both natural and anthropogenic sources has led to significant bioaccumulation in humans over the past few decades [10–12]. Human exposure studies suggest that several OH-PBDEs are present in serum at concentrations similar to, and sometimes higher than, those of parent PBDEs [13, 14]. Of concern for early neurodevelopment, PBDE and OH-PBDE levels have been shown to be especially high in young children compared to adults, likely due to mobilization of the compounds through breastmilk and high exposure rates from household products and dust [15, 16]. Concerns of such bioaccumulation are exacerbated by the ability of PBDEs to cross over the placenta, exposing developing fetuses to these toxins [17, 18]. Ominously, several lines of evidence now point towards a potential role of PBDEs in contributing to various neurodevelopmental disorders (NDDs), whose rates of occurrence have grown to alarming levels in the US [19].

The association between developmental exposures to PBDEs and NDDs has been extensively studied using mammalian models. Animals exposed to various PBDEs during prenatal and/or postnatal periods exhibit long-lasting behavioral abnormalities, including deficiencies in motor activity and cognitive functions [20–23]. Along similar lines, epidemiological studies in human populations have reported significant associations of maternal neonatal PBDE exposure with deficits in motor behavior, Intelligence Quotient (IQ) ratings (showing intellectual disability), and attention and cognitive functions in children [24–29]. Recently, an expert panel identified 70-100% probability that exposure to PBDEs contributes to IQ loss and intellectual disability that cost the EU public an estimated €9.59 billion [30]. Among other relevant findings, significant positive correlation has been established between concentration of PBDEs in postpartum breast milk and increased externalizing behavioral problems [31] (reminiscent of Attention Deficiency Hyperactivity Disorder (ADHD)-like behavior in older children), as well as between PBDE concentration in peripheral or cord blood and increased risk of attention symptoms and poorer social competency scores [32].

While there is a large amount of correlative evidence for these effects on humans, disentangling the complex mechanisms of PBDE toxicity has been a difficult task. Of the 209 PBDE congeners, a few have been the focus of research due to their prevalence. 2, 2’, 4, 4’-tetrabromodiphenyl ether (BDE-47) is among the most prevalent PBDE congeners in the environment, with a correspondingly high body burden in humans [16]. Appropriately, much research has been focused on identifying the ways in which BDE-47, and to a lesser extent, its hydroxylated metabolites, affect neural cells. Several potential mediators of the toxic effects of BDE-47 have been identified, including: 1) oxidative stress following the uncoupling of mitochondrial function by disruption of oxidative phosphorylation [33], 2) alteration of DNA methylation patterns which were associated with behavioral deficits in mice [34], 3) disruption of endocrine signalling– primarily thought to be enabled by structural similarity with triiodothyronine and thyroxine, as well as with ligands of other hormone receptors [33–37], and 4) perturbation of calcium homeostasis which has been demonstrated in several cell types including human neuronal precursors [8, 38]. Despite much progress, a description of how PBDE exposures are related to NDDs remains incomplete; it is not clear how and to what extent known molecular and cellular effects of PBDEs contribute to these adverse phenotypes.

A largely unexplored point of convergence that may help to provide an integrative explanation of the relationship between the various effects of PBDE exposures and adverse behavioral outcomes is epigenetic regulation, specifically of gene transcription during neurodevelopment. Epigenetic mechanisms are a major driving force of normal neurodevelopment [39], and many neurodevelopmental complications involve dysregulation of gene transcription [40, 41]. Although several epigenetic components of neurodevelopment are now well known, there remains much more to be characterized [42], especially in regards to how they interact with environmental challenges like PBDE exposure. Considering this, we sought to explore the ways in which exposure to BDE-47, or its hydroxylated metabolites, influences neuronal maturation and function, as well as the expression of NDD candidate genes, including many epigenetic regulators.

Methods

Plasmids and Sub-Cloning

A commercial shRNA construct for BAF170 (CCCAAACTGC TAGGGAAATTA) was obtained from Sigma. This shRNA sequence was inserted into pLKO.1-puro (designed by RNAi consortium or TRC; obtained from Addgene) and then packaged into lentiviruses. Self-inactivating HIV lentivirus particles were produced by transfecting 293 T cells with the shRNA vector, envelope (pMD2.G; Addgene), and packaging plasmids (psPAX2; Addgene) using a previously described protocol [43]. The BAF170 expression construct in a lentiviral backbone was a kind gift from Dr. Trevor Archer (NIEHS, NIH) [44]. BAF170 expression from this construct was validated by Western blotting.

Dissociated Neuronal Culture, RNAi and Cell Treatment

Cultures of cortical neurons were prepared from embryonic day 18 Sprague Dawley rats (UC Merced IACUC approval: AUP#13-0007 and AUP#16-0004). Dissociated cortical neurons were plated in Neurobasal medium (Invitrogen) supplemented with 25 μM glutamate (Sigma-Aldrich) and 0.5 mM L-glutamine (Sigma-Aldrich) and either B27 (Invitrogen) or NS21 and maintained in a similar medium without glutamate. NS21 was prepared in the laboratory as previously described [45]. Neurons were routinely used for induction assays between 10–16 days *in vitro*. For infection with recombinant lentiviruses, the viral supernatant was diluted in neuronal media and cells were infected at a multiplicity of infection ranging from 2 to 5. To induce gene transcription under basal conditions using synaptic circuits, we co-treated neurons with 50 μM Bicuculline (Sigma-Aldrich) and 75 μM 4-Aminopyridine (Acros Organics) [46]. To induce gene transcription extra-synaptically, we blocked activity with 1 μM TTX (Calbiochem) and induced the MAP-kinase pathways with 1 μM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) [47]. BDE-47 and metabolites used to treat

cultures were obtained from AccuStandard (BDE-047 N, HBDE-4003 N, HBDE-4004 N, HBDE-4005 N).

RNA Extraction and Gene Transcription Quantitation

Total RNA was isolated from dissociated neurons using the GeneJET RNA Purification Kit (Thermo) with an off-column DNase (Promega) digestion. cDNA was synthesized using MuLV reverse transcriptase (Promega), random primers (Promega), oligo dTs (Promega), and RNase inhibitors (Thermo Scientific). Quantitative real-time PCR (qRT-PCR) was performed to quantify mRNA levels of specific transcripts using iTaq Universal Sybr Green Supermix (BioRad) and the BIO-RAD CFX Connect realtime PCR Detection System. Pre-mRNA levels were estimated as previously described [43].

Sample Preparation for Electrophoresis

Neurons were lysed in ice-cold 1X RIPA buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1% Na- deoxycholate, 0.1% SDS, 0.1% NP-40) supplemented with 1: 100 protease inhibitor cocktail (Sigma-Aldrich-Aldrich). Lysed neurons were sheared by sonication (low setting; three cycles on Bioruptor®), cell debris pelleted at 15 000 rpm for 5 min at 4 °C, and clarified supernatant transferred to pre-chilled 1.5 mL microcentrifuge tube. Total cell extracts were denatured at 95 °C, for 5 min, using either homemade 5X Laemmli buffer, 2X-, or 4X-Laemmli sample buffer (both from BIO-RAD).

Western Blotting and Imaging

Denatured protein samples were resolved on 4-20%- (BIO-RAD cat. no. 4568095) or 4-15%- (BIO-RAD cat. no. 456-1083) Mini PROTEAN® gels in Tris/Glycine/SDS (BIO-RAD cat. no. 1610772). Resolved proteins were transferred onto LF PVDF membrane, using the BIO-RAD TBT RTA kit and protocol (cat. no. 1704272). PVDF membranes were incubated at 4 °C overnight with appropriate primary antibodies in 1X TBS-T with 0.5% BSA at 1: 1000 dilution. Primary antibodies included the following antibodies: β−Actin (ThermoFisher Scientific AM4302), BAF170 (CST 12760), BAF155 (CST 11956), Brg1 (CST 49360), BAF47 (CST 91735). Next day, membranes were washed three times in 1X-TBST, probed with appropriate Alexa Fluor® secondary antibodies (Life Technologies) for 40 min at room temperature, washed three times with 1X TBS-T, and imaged using BIO-RAD Multiplex ChemiDoc™ Imaging System.

Immunocytochemistry and Microscopy

Antibodies for immunocytochemistry were used at dilutions between 1: 100–1: 500 and include the following: NeuN (Millipore ABN78), Doublecortin (CST 4604 S), Nestin (Invitrogen MA1-91657). Neurons were washed twice with 1X ice-cold PBS (Fisher Sci). The cells were then incubated with 4% paraformaldehyde (Sigma-Aldrich) in 1X PBS for 15 min at room temperature and then washed twice with 1X PBS, permeabilized at room temperature for 20 min with 0.5% Triton X-100 (Fisher Sci), washed twice and blocked for 30 min with 10% goat serum (Gibco) in 1X PBS. Cells were incubated at 4 °C overnight in 3% goat serum in 1X PBS with primary antibodies. Next day, primary antibody solution was removed and cells were washed thrice with 0.05% Tween (Fisher) in 1X PBS (0.05% PBS-T), and incubated with appropriate Alexa Fluor® secondary antibody (Lifetech) for 45 min, washed thrice with 0.05% PBS-T, cured overnight with Prolong Anti-Fade Gold with DAPI and imaged. Images were captured with a Keyence BZ9000-E microscope at 40X magnification.

Cell Viability Assay

Cell viability was assessed by an MTT assay (Biotium) [48] wherein mitochondrial activity is detected colorimetrically following incubation of cells with a tetrazolium salt. The assay was conducted according to manufacturer's instruction, except that MTT incubation time was shortened to 30 min to avoid reaching a plateau where differences in product formation would be indistinguishable and reagent volumes were proportionally scaled up to appropriate amounts for 24-well plates.

Statistics

Error bars represent standard error of mean throughout this article. Statistical comparison of datasets was performed by one way ANOVA with Fisher[CVOAPS]s LSD (Figs 2C and 6A) or by two way ANOVA with Tukey[CVOAPS]s post hoc test (all other figures). Biological replicates are indicated throughout as *N* in corresponding figure legends. Biological replicates constitute cell culture preparations from independent dams.

Results

Characterization of E18 Primary Rat Neuronal Cultures

We dissected brain tissue from the pups of timed-pregnant Sprague-Dawley rats (*Rattus norvegicus*) on embryonic day 18 (E18) to obtain dissociated cortical neurons. Cells were then plated as monolayers in supplemented Neurobasal growth medium. The identity of these cultured cells was characterized over the first week of growth by immunocytochemistry (ICC) using antibodies raised against Nestin, DCX, and NeuN (neural progenitor, differentiating neuron, and differentiated neuronal markers, respectively) (Fig. 1) [49–51]. The cultures were composed of cells exhibiting expression of: Nestin and DCX on day 0 *in vitro* (DIV 0), predominantly DCX expression by DIV 4, and largely NeuN expression by DIV 7. Based on the expression of these markers, we identified our DIV 0 cells to be a mixture of both neuronal progenitors and differentiating neurons, whereas DIV 4 and DIV 7 cells were predominantly differentiating and differentiated neurons respectively. Utilizing cultures of this nature allowed us to investigate the effects of exposure to BDE-47 and its hydroxylated metabolites across various early stages of neuronal maturation *in vitro*.

6OH-BDE-47 Impairs Neuronal Maturation and Activity-Induced Gene Transcription

Mature neurons sense environmental cues through neuronal activity and can respond via activity-induced gene transcription [52]. Therefore, we sought to test the effects of PBDE exposure on functional maturation by conducting neuronal activity-induced gene transcription assays using a widely studied, neuron-specific immediate early gene (IEG), *Arc*, as a readout (referred to from here on as 'activity-induced gene transcription assay'). Assays were conducted in cultures that were chronically exposed to 1 μM of BDE-47 or one of its hydroxylated metabolites– 3OH-BDE-47 (3OH), 5OH-BDE-47 (5OH), and 6OH-BDE-47 (6OH) and neuronal activity was induced using Bicuculline and 4AP (Bic + 4AP). *Arc* pre-mRNA, the direct output of transcription, was detected at 15 min after induction and quantified by quantitative real-time PCR (qRT-PCR) using intron-exon

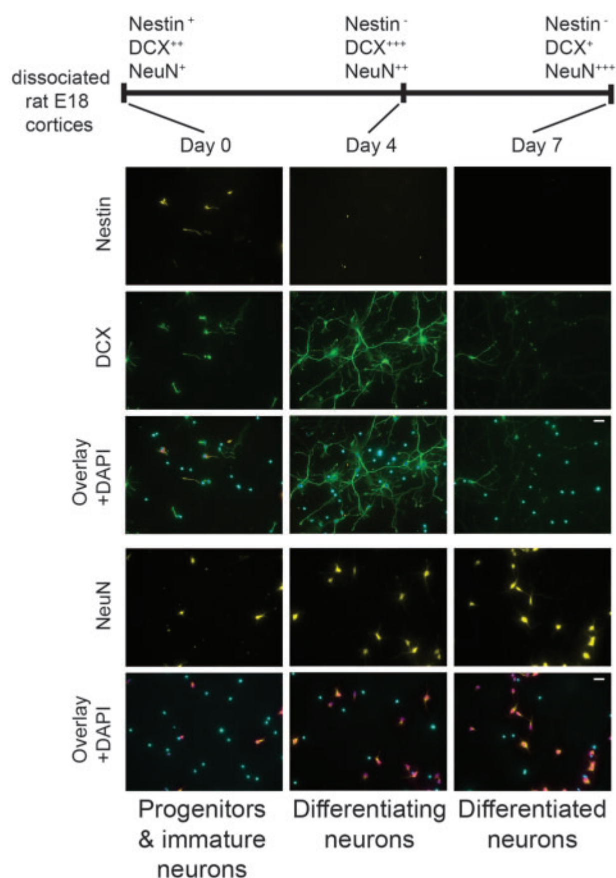

Figure 1: Characterization of neuronal cultures across the first week of growth by immunocytochemistry. Rat cortical neurons were obtained by dissecting and dissociating cortices from E18 pup brains. Cultures derived from these cells were stained using antibodies against several markers of neuronal maturation: Nestin (neuronal precursor), DCX (differentiating neurons), and NeuN (differentiated neurons). Staining was conducted at indicated time points. Scale bar = 25μm. *N*=3

spanning primers. We found that transcription of *Arc* was significantly attenuated in cultures exposed to 6OH from DIV 0-10, but not in cultures exposed to other compounds (Fig. 2B) suggesting that chronic exposure to 6OH may interfere with functional maturation of neurons.

Lack of functional maturation could be an inadvertent fallout of 6OH-induced neurotoxicity or impaired neuronal differentiation. Any cytotoxicity in cells exposed to different doses of 6OH was measured by MTT assay. This range of doses was selected based on an approximation of environmentally relevant exposures. The reasoning and justification for this approximation is detailed further in the discussion. While 1 μM 6OH was found to induce a significant reduction in cell viability after chronic exposure for ten days, 0.5 and 0.1 μM 6OH doses did not (Fig. 2C). Next, we studied the effect of 6OH on neuronal differentiation. 6OH has been previously shown to inhibit differentiation at lower concentrations than its parent compound in adult neural progenitor cells and human neuronal precursors [53, 54]. To verify any effect of 6OH on neuronal differentiation in our system, we stained for neuronal fate markers (NeuN and Tuj1) in DIV 7 neurons after prolonged exposure to the non-cytotoxic 0.5 μM 6OH dose (Fig. 2D–F). On DIV 7, when neurons are differentiated in our system (Fig. 1), there was no difference in fraction of NeuN positive neurons between untreated and 6OH treated cultures (Fig. 2D and E). Similar results were obtained with Tuj1, another marker for differentiated neurons (Fig. 2F), indicating that neurons can attain their fate and differentiate irrespective of exposure to 6OH in our culture conditions. Therefore, reasons for 6OH exposure-related lack of functional maturation (Fig. 2B) likely lie elsewhere.

Next, to explore any differences in 6OH effects between differentiating and differentiated neurons, we conducted additional activity-induced gene transcription assays, exposing cells to a range of 6OH concentrations from both DIV 0 and DIV 7. From these assays, a dose-dependent activity-induced *Arc* transcriptional response was observed in cultures exposed at both DIV 0 and DIV 7 (Fig. 2G and H). Interestingly 0.5 μM 6OH exposure, which does not significantly affect cell viability, produced effects that were not statistically different from 1 μM exposure. Taken together, these results indicate that chronic 6OH exposure impairs functional neuronal maturation while differentially affecting cell viability depending on the exposure dose.

6OH-BDE-47 Exposure Impairs Synaptic and Extra-Synaptic Modules of Activity-Induced Transcription

Transcription of IEGs in response to neuronal activity relies on a cascade of intracellular components starting with functional synapses, then transduction of signals from the synapse to the nucleus, and finally chromatin accessibility in the nucleus itself [55, 56]. As Bic + 4AP treatment induces neuronal activity by GABAergic disinhibition, any of these factors affected by 6OH could be contributing to the decreased production of nascent *Arc* pre-mRNA in Figure 2. As such, results from Bic + 4AP induction assays potentially reflect an accumulation of effects arising from dysregulation at any point from the synapse to the nucleus. Therefore, to more specifically test if 6OH was acting at synapses or further downstream, we induced *Arc* transcription intracellularly at the signal transduction level by stimulating the Mitogen Activated Protein Kinase (MAPK) pathway, which is known to transduce signals for rapid neuronal gene transcription [57, 58]. Our laboratory has established that neuronal rapid IEGs, including *Arc* [43], may be induced extrasynaptically by activating the MAPK pathway with 1 μM of the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) while blocking neuronal activity with 1 μM tetrodotoxin (TTX), a potent sodium channel blocker (Fig. 3A) [59]. Using this extrasynaptic induction protocol, we found that *Arc* is induced within 15 min. This *Arc* pre-mRNA induction was inhibited significantly by 1 μM, but not 0.5 μM or 0.1 μM, 6OH exposure (Fig. 3C). Although 1 μM 6OH exposure caused a certain amount of cell death (Fig. 2C), the effect of the same dose seen in this assay may not be entirely due to neurotoxicity because the *Arc* induction values are normalized by an internal control (*Gapdh*, representing live cells). Additionally, comparing Figs 2G and 3C, our data indicate that prolonged exposure to the non-lethal dose of 6OH (0.5 μM) may impair synaptic formation and/or functions (Bic + 4AP assays; Fig. 2G), but spares extra-synaptic cascades of events that lead up to *Arc* transcription (TTX + PMA assays; Fig. 3C).

One major suspected mode of action for PBDE toxicity is dysregulation of thyroid hormone homeostasis, including disruption of thyroid hormone levels, transport, and receptor activity [4]. These effects are thought to be mediated by structural similarity between PBDEs and thyroid signaling molecules [7]. To

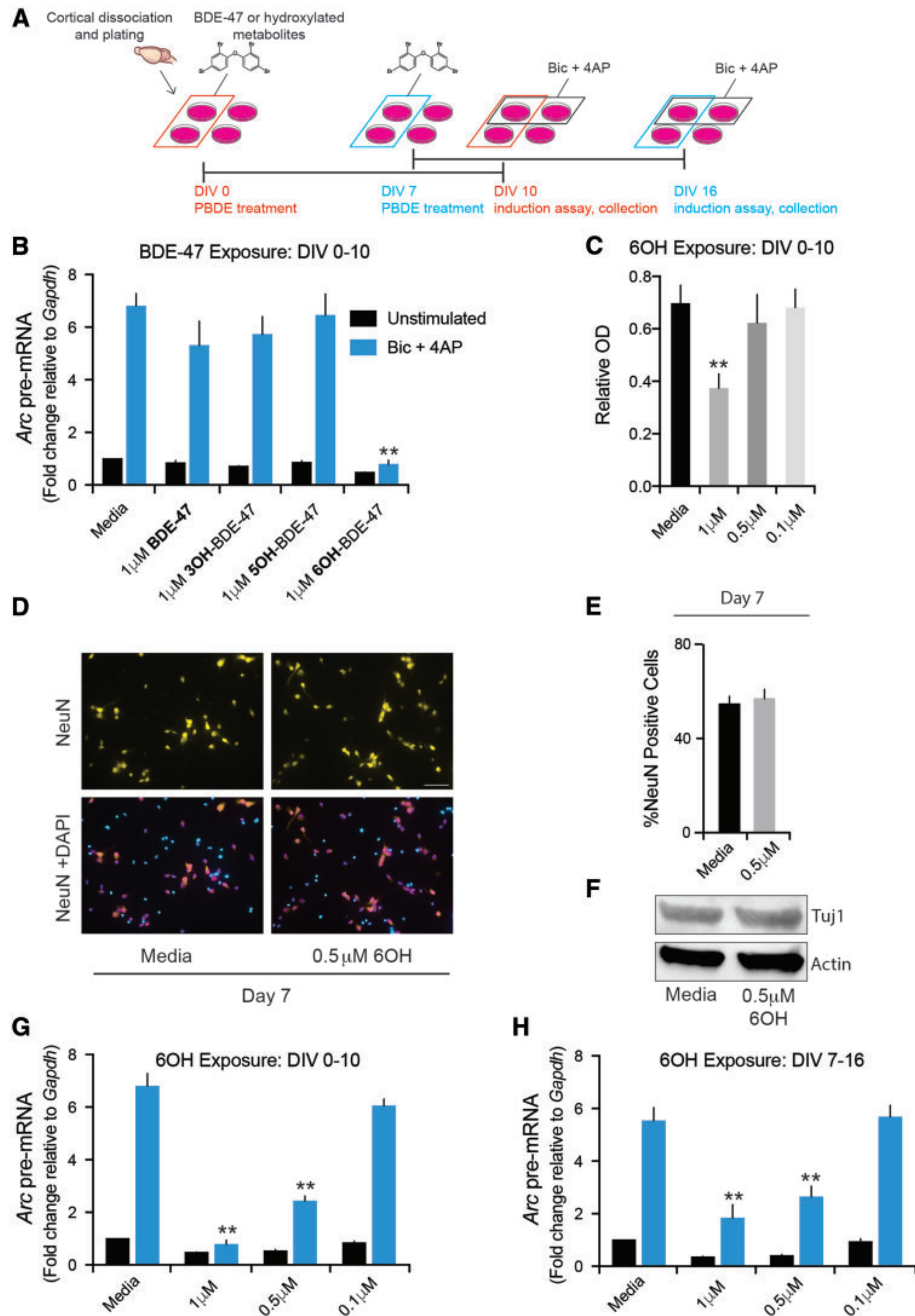

Figure 2: Chronic 6OH exposure is detrimental to neuronal maturation and function. Activity induced *Arc* expression assays were conducted by treating cultures with Bic and 4AP for 15 min. (**A**) Timeline depicting PBDE exposure durations leading up to activity induction assays. Ten day exposures were started either concurrently with plating (DIV 0) or after a week of growth (DIV 7) to assess differences in effect on differentiating versus differentiated neurons. (**B**) Induction of *Arc* after a 10-day exposure to 1μM BDE-47 or one of its hydroxylated metabolites, $N=3$. (**C**) Assessment of cell viability following 10 days of 1μM 6OH-BDE-47 exposure. Viability was assessed colorimetrically by MTT assay. $N=3$. (**D**) Representative images of NeuN and DAPI stained cells on DIV 7 following treatment with 0.5μM 6OH starting at DIV 0. Scale bar = 50μm (**E**) Quantification of NeuN and DAPI staining represented in D, (2, 562 untreated and 2, 634 6OH treated cells counted). (**F**) Representative western blot for Tuj1 (a marker of differentiated neurons) in cells exposed to 0.5μM 6OH-BDE-47 for seven days, $N=3$. (**G**) Induction of *Arc* after a ten-day exposure to various concentrations of 6OH, $N=3$. (**H**) Similar assay as shown in (**G**) except 6OH exposure was started on DIV 7, $N=4$. * $P < 0.05$, ** $P < 0.01$. ns = non-significant

test if the observed effects of 6OH in our assays are mediated primarily by disruption of intracellular thyroid signaling, cultures were co-exposed to 1 μM 6OH and either 30 or 3 nM triiodothyronine (T_3), which is present at low levels in culture media already. Elevated levels of T_3 were shown to rescue 10 μM BDE-47-induced reduction of neuronal migration in another study [53]. In our assays, elevated levels of T_3 did not rescue 1 μM 6OH-induced attenuation of *Arc* induction following Bic + 4AP

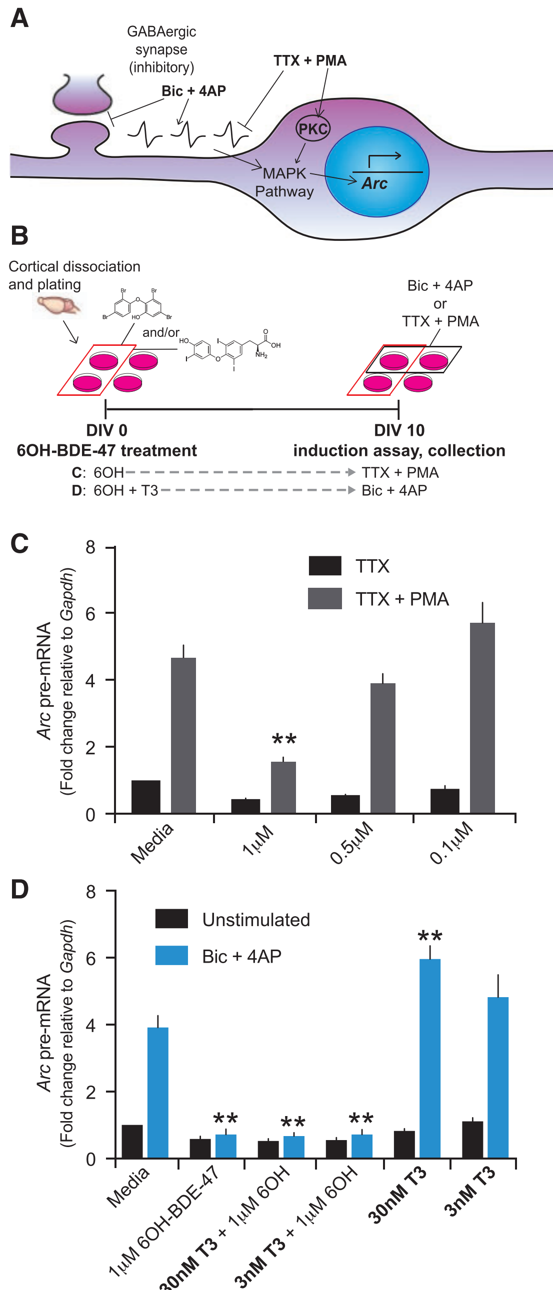

Figure 3: Effects of 6OH exposure are at the synaptic and extrasynaptic level and are not primarily mediated by disruption of thyroid hormone signaling. Activity induced *Arc* expression assays were conducted by treating cultures with Bic and 4AP or TTX and PMA for 15 min after ten days of 6OH exposure starting at the time of plating. (**A**) Depiction of different modes of gene transcription assays. Note: Bic+4AP induction (Fig. 2) is reliant on synaptic activity whereas TTX+PMA induction bypasses synapses and jumpstarts signaling cascades extra-synaptically. (**B**) Timeline depicting 6OH exposures with or without addition of triiodothyronine (T3) followed by various activity induction assays. (**C**) Induction of *Arc* via direct activation of the MAPK pathway with PMA while blocking membrane activity with TTX in cultures exposed to various concentrations of 6OH N=5. (**D**) Induction of *Arc* with Bic+4AP following exposure to 6OH with and without additions of T3 to outcompete potential interactions of 6OH with thyroid hormone transporters and receptors N=3. *$P < 0.05$, **$P < 0.01$

stimulation, although the addition of 30 nM T_3 alone did significantly enhance induced *Arc* transcription (Fig. 3D). Together, these results indicate that boosted levels of thyroid hormone cannot rescue effects of 6OH exposure, perhaps including its neurotoxic effects, opening the possibility of a thyroid-hormone pathway-independent mechanism of 6OH toxicity.

BDE-47 and Its Hydroxylated Metabolites Dysregulate Expression of NDD Candidate Genes

Observing that chronic exposure to 6OH disrupts neuronal activity-related function in dosage-dependent manners, and that the effects are not primarily mediated by disruption of thyroid hormone homeostasis, we sought to identify other potential mechanisms. To accomplish this, we screened 15 NDD candidate genes, coding for proteins of various functions important for neuronal development and function (Table 1), for changes in mRNA levels following exposure to 1 μM BDE-47 or its hydroxylated metabolites. Exposures were started at various time points to assess effects during neuronal differentiation. We found that exposure to BDE-47, 3OH, and 5OH altered mRNA levels of only a few genes, while 6OH exposure altered mRNA levels of 11 out of 15 genes at the earliest and middle time points (exposures at DIV 0 and DIV 4), and a smaller subset when differentiated neurons were exposed starting at DIV 7 (Fig. 4). This observation suggests that 6OH exposure may have temporally specific effects, with immature neurons being affected to a seemingly greater extent. Following up on the widespread effect caused by 6OH, we measured mRNA levels of the same 15 genes after exposure to a range of 6OH concentrations over the various time points (Fig. 5). Dysregulation of mRNA levels by 6OH was found to be dose-dependent, with transcripts of a few genes exhibiting significant changes at concentrations as low as 10 nM (*Arid1b*, *Tbl1xr1*, and *Adnp*, data not shown). Of particular interest are *Arid1b* and *Smarcc2*, both of which code for subunits of the BAF chromatin remodeling complex [60], as well as *Shank3*, a high confidence autism candidate gene whose protein product is involved in the organization of the postsynaptic density [61]. In our screen, *Shank3* was significantly dysregulated by 6OH in an opposing manner depending on the time of exposure (Figs 4 and 5). Together, these screens demonstrate dysregulation of mRNA levels of several NDD candidate genes by chronic 6OH exposure in a mammalian model, and further validate the dose- and time-of-exposure dependence of 6OH toxicity. They also provide many potential novel targets for further investigation.

While changes in mRNA levels following 6OH exposure can be informative of altered cellular states, they do not necessarily indicate direct effects on the transcription of target genes. This is due to mRNA abundance being a function of the rate of transcription as well as of transcript decay, which can vary greatly [62, 63]. To verify if 6OH-induced changes to mRNA levels were due to a direct effect on the transcription of target genes, we designed primers to detect pre-mRNAs, the short-lived, direct product of transcription by RNA polymerase II. We focused on genes strongly affected in differentiating neurons (cultures with exposure starting on DIV0), and measured their respective pre-mRNA levels at time points within that window of exposure, where early neuronal differentiation is occurring (DIV0-5). Interestingly, as revealed by MTT assays and unlike what is seen in differentiated neurons (Fig. 2C), 1 μM 6OH did not negatively impact cell viability by DIV3 (Fig. 6A). In these cells, as revealed by pre-mRNA assays, 6OH exposure impacted most genes only at the mRNA level, perhaps via indirect mechanisms that influence mRNA half-lives. However, expression profiles of three of the tested genes were found to be significantly dysregulated at the pre-mRNA level – intriguingly, all of which encode BAF complex subunits or interacting proteins (*Smarcc2, Arid1b, and Adnp*). (Fig. 6B).

Table 1: Neurodevelopmental disorder candidate genes screened for mRNA dysregulation following chronic exposure to BDE-47 or its hydroxylated metabolites

Gene	Description/Function	Associated Brain Disorders
Adnp	Activity-Dependent Neuroprotective Protein: zinc finger transcription factor that modulates p53 activity and interacts with BAF chromatin remodeling complexes to regulate transcription	ASD [81], ID [82]
Arid1b	AT-Rich Interaction Domain 1B (BAF250b): DNA interacting protein, subunit of the neural precursor and neuron specific BAF chromatin remodeling complexes	ASD [83], ID [82]
Cbx4	Chromobox 4: component of a Polycomb group (PcG) PRC1-like complex which is involved in transcriptional repression of many genes throughout development and mediates monoubiquination of histone 2A lysine 119 residues	ASD [84]
Ctnnb1	Catenin Beta 1: component of the canonical Wnt signaling pathway, coactivator of TCF/LEF transcription factors that activate Wnt responsive genes which play diverse neurodevelopmental roles such as central nervous system patterning, and neural stem and precursor cell proliferation	ASD [81], ID [82]
Dyrk1a	Dual Specificity Tyrosine Phosphorylation-Regulated Kinase 1A: a nuclear protein kinase that catalyzes autophosphorylation of serine/threonine and tyrosine residues, thought to play a role in regulating signaling pathways contributing to cell proliferation and neurodevelopment	ASD [81], ID [82]
Med13l	Mediator Complex Subunit 13 Like: a subunit of the mediator complex, a transcriptional coactivator for RNA polymerase II transcribed genes that is recruited to promoters by binding with regulatory proteins thus serving as a scaffold for the preinitiation complex, involved in early neurodevelopment	ASD [84], ID [82]
Ncor1	Nuclear Receptor Corepressor 1: corepressor of thyroid-hormone and retinoic-acid receptor target genes, known to recruit histone deacetylases that promote the formation of condensed chromatin structure that prevents access of transcription factors and thus represses transcription	ASD [84], HD [85]
Npas2	Neuronal PAS Domain Protein 2: transcription factor that is a core component of the circadian clock, also known to regulate the transcription of metabolism, cell cycle, and DNA repair related genes	ASD [86]
Phf2	PHD Finger Protein 2: a zinc-finger-like plant homeodomain containing demethylase that acts on both histone and non-histone proteins, dimerizes with Arid5b at target promoters and demethylates histone 3 lysine 9 (H3K9me2) residues leading to transcriptional activation	ASD [84]
Rps6ka2	Ribosomal Protein S6 Kinase A2: a member of the RSK family of serine/threonine kinases that phosphorylates members of the mitogen-activated protein kinase signalling pathway, regulates processes such as cell growth, survival, and proliferation	ASD [87]
Shank3	SH3 and Multiple Ankyrin Repeat Domains 3: major scaffold protein of the postsynaptic density that organizes neurotransmitter receptors, ion channels, and other membrane proteins via interactions with the actin cytoskeleton, plays a role in dendritic spine maturation as well as synaptic formation and plasticity	ASD [88], ID [89], SCZ [90]
Smarcc2	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin Subfamily C Member 2 (BAF170): subunit of the neural precursor and neuron specific BAF chromatin remodeling complexes, thought to be a scaffolding subunit required for stabilization of other BAF subunits, involved in transcriptional activation and repression, known to regulate cortical thickness and neural progenitor proliferation	ASD [91], ID [92], SCZ [41]
Sp1	Specificity Protein 1: a zinc finger transcription factor that binds GC-rich motifs at many promoters, involved in many cellular processes such as differentiation, growth, apoptosis, and recruitment of chromatin remodeling enzymes including Brg1 and Brg associated factors to regulate transcription	ASD [93]
Tbl1xr1	Transducin Beta Like 1 X-Linked Receptor 1: thought to be a component of the NCoR and HDAC3 repressive complexes, and is required for transcriptional activation by a variety of transcription factors	ASD [81], ID [94]
Ubr3	Ubiquitin-Protein Ligase E3-Alpha-3: targets proteins for degradation by ubiquinitination, may be involved in chromatin regulation and transcriptional silencing (by similarity)	ASD [81]

Abbreviations: ASD- autism spectrum disorder, ID- intellectual disability, SCZ- schizophrenia, HD- Huntington[CVOAPS]s disease

6OH-BDE-47 Dysregulates Expression of *Smarcc2* (BAF170) and Other BAF Complex Subunits

One of the genes exhibiting 6OH-induced changes in pre-mRNA level, *Smarcc2*, codes for BAF170, a core subunit of the mammalian SWI/SNF (SWItch/Sucrose Non-Fermentable)-like BAF complex. BAF complexes are ATP-dependent chromatin remodelers that play important and fundamental roles in driving neurodevelopmental processes [64]. BAF170 is thought to act as a scaffolding subunit that is critical for the stability of these complexes [65]. Therefore, to test the effect of 6OH on expression of other BAF subunits, we screened mRNA levels of other known subunits of the neuronal precursor and neuron specific

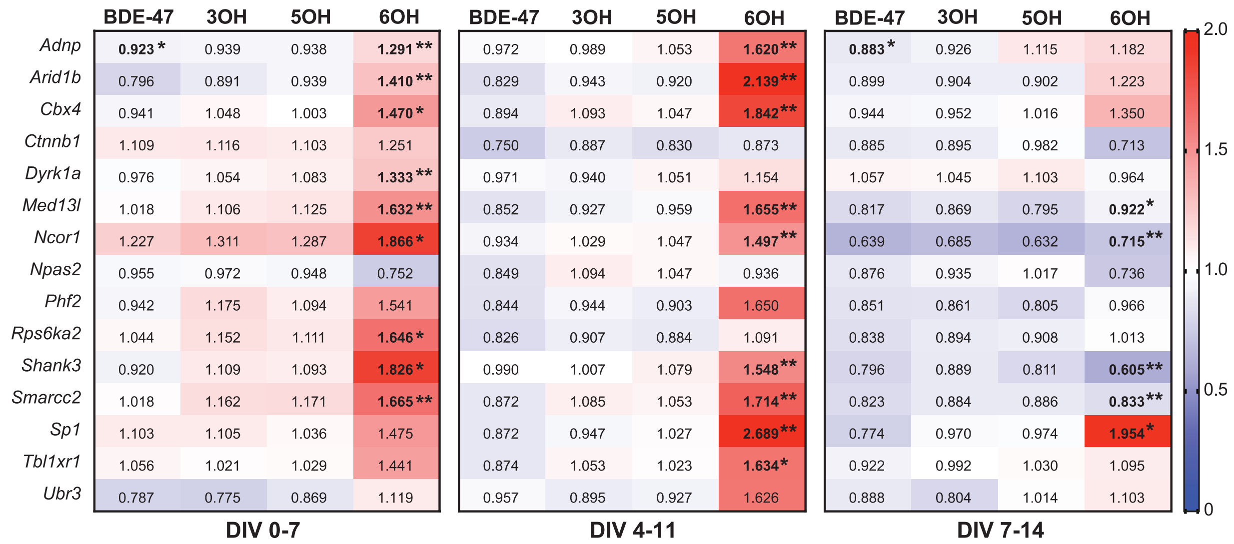

Figure 4: Exposure to BDE-47 and its hydroxylated metabolites dysregulates mRNA levels of neurodevelopmental disorder candidate genes. 15 neurodevelopmental disorder-associated genes screened for changes in mRNA abundance by qRT-PCR following exposures to 1μM BDE-47 or one of its hydroxylated metabolites for periods indicated below each heatmap. Each row depicts changes in mRNA level for a gene while each column reflects exposure to a different compound. Results are displayed as fold change relative to control. Cells with a fold change above two have saturated coloring. * $P < 0.05$, ** $P < 0.01$. N=3–5

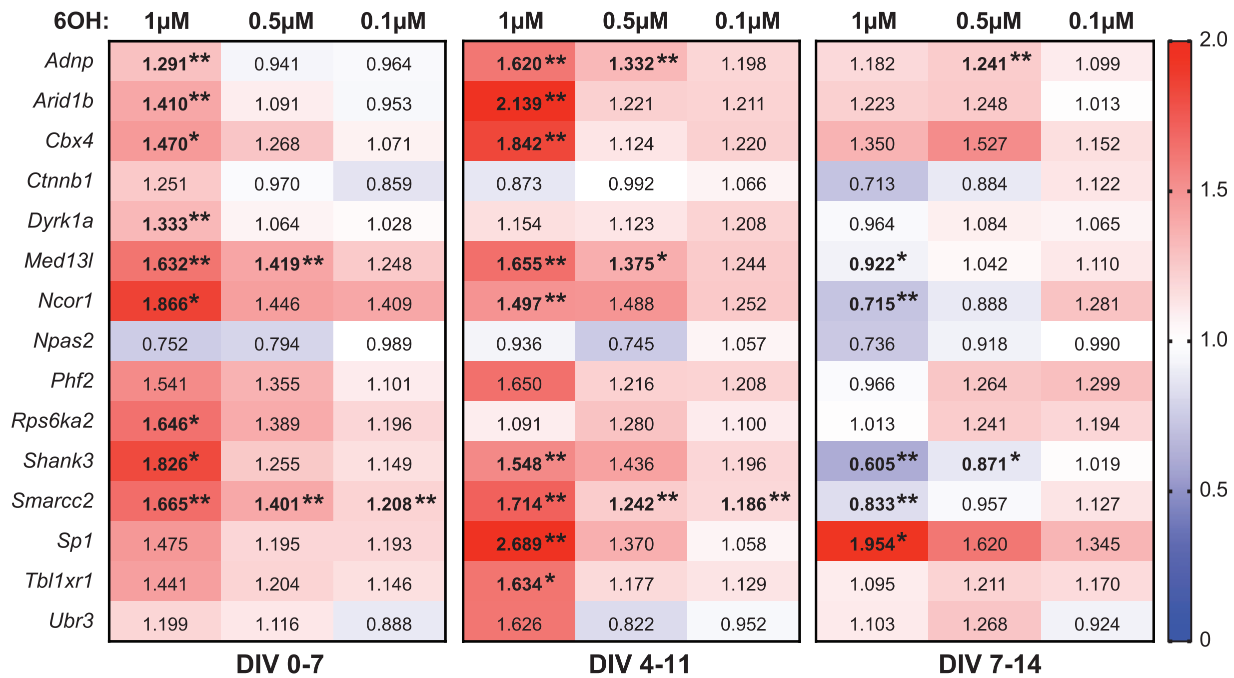

Figure 5: Dysregulation of neurodevelopmental disorder candidate gene mRNA levels by 6OH is dose- and time-of-exposure-dependent. 15 neurodevelopmental disorder-associated genes screened for changes in mRNA abundance by qRT-PCR following exposures to various concentrations of 6OH for periods indicated below each heatmap. Each row depicts changes in mRNA level for a gene while each column reflects exposure to a different concentration of 6OH. Results are displayed as fold change relative to control. Cells with a fold change above two have saturated coloring. * $P < 0.05$, ** $P < 0.01$. N=3–4

BAF complexes (npBAF/nBAF) and found significant upregulation of several additional subunits, namely, Brm, Brg1, BAF60a, BAF53a, and BAF47 (Fig. 7A). Focusing on BAF170, we then sought to confirm 6OH-induced dysregulation of this major BAF subunit at the protein level by Western blotting. Here, to our bewilderment, we encountered two opposing outcomes with almost equal frequency across replicates. In some trials, chronic exposure of differentiating neurons to 0.1-1 μM 6OH downregulated BAF170 protein levels. In others, BAF170 protein levels were upregulated by the same treatment (Fig. 7B). Initially baffling, such vacillating outcomes may be explained if expression of BAF170 oscillates during neuronal differentiation and 6OH exposure shifts the oscillation frame (Fig. 7B). This hypothesis is supported by previous work that found the expression of BAF170 to be very dynamic across mouse corticogenesis [66] and biphasic in H1 embryonic stem cells [44]. To test this possibility, we collected samples at 6 h intervals after plating for the first 48 h and then assessed BAF170 protein levels by western blotting. As shown in Fig. 7C (quantified in Fig. 7D), BAF170 levels were found to oscillate with time. Next, we attempted to

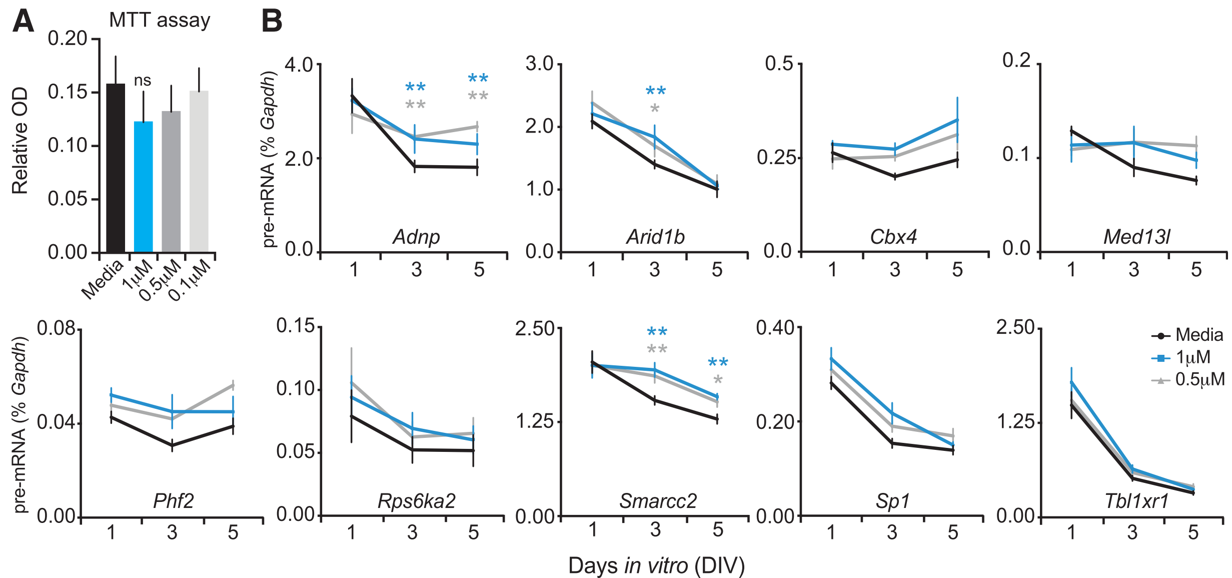

Figure 6: Exposure to 6OH dysregulates pre-mRNA levels of a subset of genes affected at the mRNA level. (A) Assessment of cell viability following 3 days of exposure to 1µM 6OH. Viability was assessed colorimetrically by an MTT assay. N=3. (B) A subset of neurodevelopmental disorder associated genes found to have dysregulated mRNA levels following 6OH exposure were screened for changes in pre-mRNA abundance which better reflect effects on transcription of a given gene. Time points within the earliest exposure period (DIV 0-7) were chosen to more clearly observe direct effects of 6OH toxicity. *P < 0.05, **P < 0.01. N=3–7

quantify such oscillation with and without 6OH exposure. To our surprise, these oscillations varied greatly between biological Ns, occluding any statistical analysis after averaging data from multiple trials. Despite such variations, 6OH treatment consistently altered the oscillatory pattern in every N (5 attempted; three displayed in Fig. 7E), providing support to our 'altered BAF170 oscillation' hypothesis (Fig. 7B).

These BAF170 protein data, alongside its altered pre-mRNA and mRNA levels, and the observed alterations to mRNA levels of other BAF subunits, indicate that 6OH exposure likely influences BAF complex composition/stability and, therefore, potentially impacts BAF-dependent functions during neuronal differentiation, including chromatin remodeling and regulation of gene transcription.

Role of BAF170 in Neuronal Maturation and Activity Induced *Arc* Transcription

Finally, to directly study effects of BAF170 dysregulation on functional maturation of neurons, as would be expected due to 6OH-induced oscillation, we bi-directionally altered its levels in developing cells (DIV 0-4) by either overexpressing BAF170, or depleting it via RNAi (lentivirus-mediated delivery of BAF170 shRNA). Both methods were validated by western blot (Fig. 8D and G). We then performed synapse-dependent and synapse-independent *Arc* induction assays. The phenotypic outcomes of these assays were unaffected by overexpression of BAF170 (Fig. 8B and C). Interestingly, depletion of BAF170 impaired *Arc* induction in the synapse-dependent Bic + 4AP assay (Fig. 8E), but not in the synapse-independent PMA + TTX assay (Fig. 8F). This observation is reminiscent of the effects of lower 6OH doses on *Arc* induction using these protocol (Figs 2 and 3D). Additionally, we found that depletion of BAF170 reduces protein levels of other BAF subunits (Fig. 8G). These observations support the idea that BAF170 acts as a scaffolding component in BAF complexes and indicates that dysregulation of BAF170 may effectively alter BAF complex stability and composition (Fig. 7A) [65]. Interestingly, BAF155 protein levels did not decrease while other subunits, namely BAF60a and BAF47 whose mRNA levels were significantly upregulated following 6OH exposure (Fig. 7A), exhibited reduced protein levels following loss of BAF170 by RNAi (Fig. 8G).

Taken together, our data suggest that BAF170 depletion, not its overexpression, likely impairs neuronal maturation via synapse-dependent mechanisms, but not extra-synaptic components of activity-induced gene transcription. As effects of BAF170-KD are highly evocative of the effects of 6OH exposure, we propose that environmentally-relevant doses of 6OH may act via dysregulation of the BAF complex, especially BAF170, to interfere with neuronal maturation and function.

Discussion

In this study, we have demonstrated several novel aspects of PBDE neurotoxicity. Initially, we show the effects of chronic nanomolar range exposures to BDE-47 and its hydroxylated metabolites on neuronal activity-induced gene transcription and functional maturation of primary embryonic rat cortical neurons. We then demonstrate how similar exposures impact transcription of NDD candidate genes by quantification of mRNA and pre-mRNA levels. One of the strongest candidates from our screen, *Smarcc2*, then prompted us to focus on the effects of 6OH exposure on BAF170 expression and, preliminarily, on BAF170-related regulation of neuronal maturation by measuring levels of activity-induced *Arc* expression. Together, our results indicate that exposure to the hydroxylated BDE-47 metabolite 6OH strongly influences functional maturation of neurons, potentially independent of thyroid hormone disruption mechanisms– perhaps instead exerting effects mediated in part at the level of epigenetic regulation via disruption of BAF chromatin remodeling complexes.

Our utilization of the activity-induced transcription of an immediate early gene (*Arc*) as a readout of functional neuronal maturation is, to the best of our knowledge, a novel approach in assessing the neurotoxicity of PBDEs. Immediate early gene induction is a strong tool to this end as it relies on several

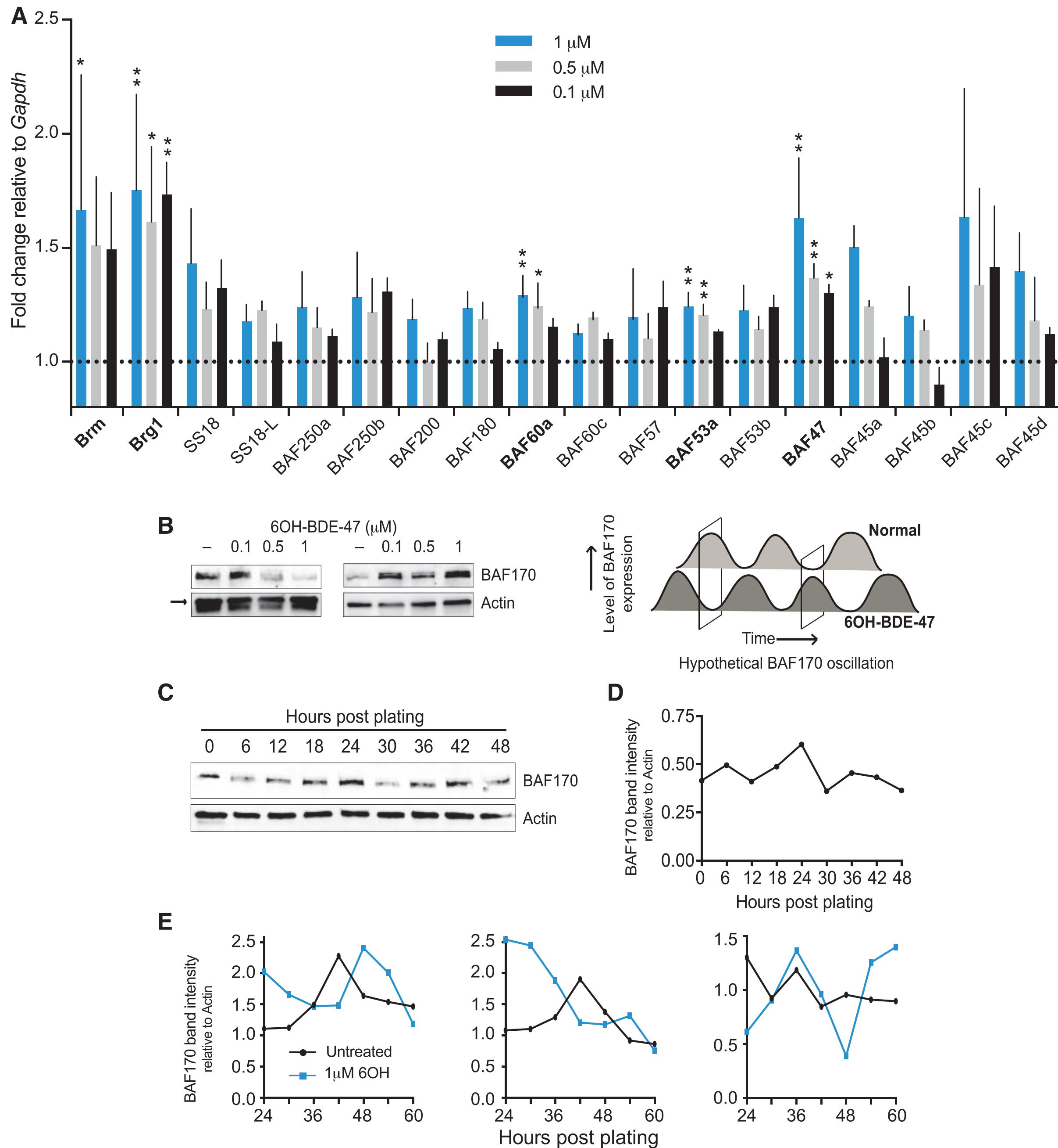

Figure 7: BAF170 protein levels and mRNA levels of BAF complex subunits are dysregulated by 6OH exposure. (**A**) Abundance of various BAF protein mRNAs estimated by qRT-PCR following exposure to various concentrations of 6OH from DIV 0-7, N=3–4. (**B**) Top: BAF170 protein levels observed by western blotting following 48 h of exposure to various concentrations of 6OH. N=6. Bottom: opposing outcomes can be explained if BAF170 is expressed in an oscillatory manner and 6OH exposures disrupt regulatory mechanisms shaping the temporal expression patterns. (**C**) Time-course western blot for BAF170 with samples collected every 6 h after plating for the first 48 h. (**D**) Quantification of BAF170 band intensities from blot shown in (**C**) as an example and evidence of BAF170 oscillation in normal cells. (**E**) Quantification of similar time-course experiments as in (**C**), with collections every 6 h starting 24 h after plating. Note the inherent variation in oscillatory pattern. Cells were either untreated or exposed to 1 μM 6OH at the time of plating. N=4 (3 represented) *P < 0.05, **P < 0.01. Note: Because of inherent variation in the oscillatory pattern, averaging these data sets 'flatten' out the phenotype and is therefore not attempted

critical cellular mechanisms that are established across neuronal maturation, ranging from proper synaptic function to nuclear regulation of gene transcription (Fig. 3A), allowing us to broadly assess the extent of PBDE-induced effects on functional neuronal maturation. Further, inducing *Arc* expression both synaptically (Bic + 4AP) and extra-synaptically (TTX + PMA) allowed us to hone in on where these neurotoxic effects are being exerted. These assays reveal that 6OH-BDE-47 interferes with neuronal maturation likely at the synaptic level.

As mentioned previously, the range of PBDE doses used in this study were estimated based on prior work done in mammalian models to approximate relevant concentrations observed in the brains of exposed animals. To determine this range, an exhaustive review was conducted of literature reporting levels of BDE-47 accumulation in rodent brain following either chronic

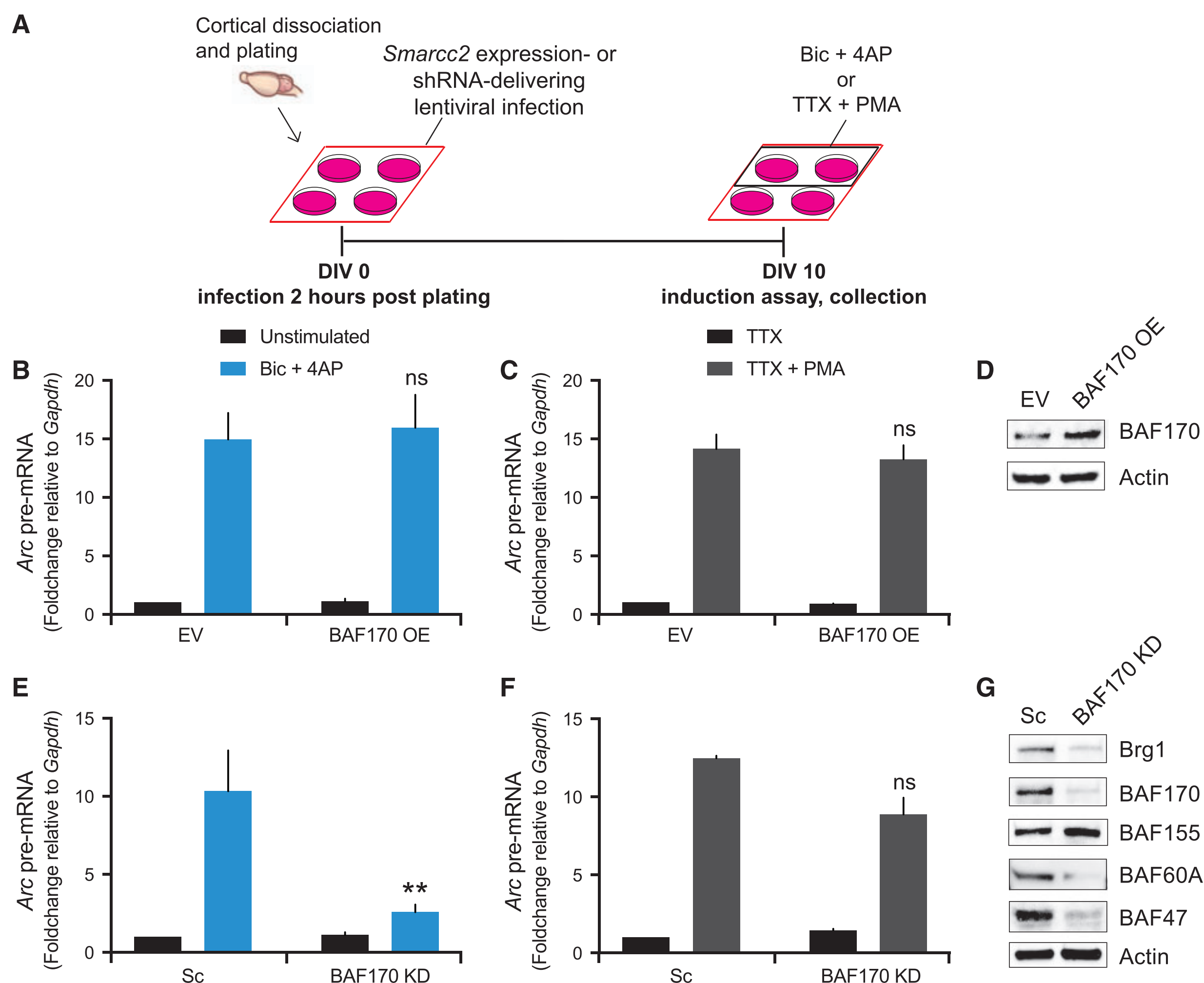

Figure 8: BAF170 contributes to the stability of BAF subunits and proper circuit formation, but is not required for MAPK stimulated *Arc* transcription. (**A**) Depiction of the timing of lentiviral mediated manipulation of BAF170 levels and subsequent activity induced *Arc* induction assays. (**B, C**) *Arc* induction following Bic+4AP or TTX+PMA stimulation in cells overexpressing BAF170, N=3. (**D**) Representative western blot validating BAF170 overexpression construct. (**E,F**) *Arc* induction following Bic+4AP or TTX+PMA stimulation in cells depleted of BAF170, N=3. (**G**) Representative western blots demonstrating the effect of BAF170 knockdown on protein levels of other BAF complex subunits, N=3. **$P < 0.01$. ns = non-significant

or single dose administration [67–73]. After converting the commonly reported unit of ng/g of toxin to nmol/L (converting from lipid concentration to molarity), the average concentration of BDE-47 was determined to be 597 nM. Studies investigating the *in vitro* effects of PBDEs have often been conducted using concentrations in the low to mid micromolar range. Summarizing human studies, the USEPA's 2010 report assessing the environmental prevalence of PBDEs estimated the body burden of total PBDEs in the general population to be 30-100 ng/g (approximately 60 to 200 nM), with toddlers tending to have higher body burdens compared to older children and adults. Importantly, BDE-47 alone was estimated to account for 50% of total PBDE concentrations [16]. It has also been reported that hydroxylated metabolites of PBDEs accumulate in human serum and are nearly as abundant (approximately 45% of total detectable PBDEs) as parent PBDE compounds in human fetal blood samples collected in the United States [13, 14]. Considering the reported brain accumulation levels of BDE-47 in rodents, concentrations commonly used for *in vitro* studies to date, and the prevalence of BDE-47 and its hydroxylated metabolites in humans, we used a range of 100-1, 000 nM BDE-47 and its hydroxylated metabolites for our studies. This range spans reported accumulated concentrations in rodent brain, is low relative to previous *in vitro* studies, and is on the same order of magnitude as reported human levels of PBDEs and their metabolites.

It has been previously demonstrated that micromolar BDE-47 exposures decrease both neuronal and oligodendrocyte differentiation in human neural progenitor cells [53]. Another study found that BDE-47 and 6OH decrease neuronal and oligodendrocyte differentiation of mouse adult neural stem cells (aNSC), with 6OH producing an effect following nanomolar exposures, at concentrations approximately 20 times lower than its parent compound [54]. Using activity-induced gene transcription assays as a readout in cultured embryonic rat cortical neurons, we found that chronic nanomolar exposure to 6OH disrupts functional maturation. These effects were found to be independent of 6OH cytotoxicity or alteration of differentiation for 0.5 μM exposures. Many of the observed effects were found to be dose-dependent whereas transcriptional profiles (Figs 4 and 5) were additionally sensitive to time of exposure.

Additionally, these functional effects appear to be mediated at both synaptic and extra-synaptic levels, as evidenced by decreases in *Arc* induction following Bic + 4AP and TTX + PMA

treatments. Effects on IEG transcription by both assays indicate that 6OH, at higher doses, may act to compromise cell viability, disrupt mature synapse formation, as well as interfere with cellular mechanisms at or downstream of the MAPK pathway that regulate activity-induced *Arc* transcription (Figs 2 and 3). Interestingly, addition of excess levels of the thyroid hormone triiodothyronine (T3) did not produce any phenotypic rescue (as seen previously for BDE-47 induced impairment of neuronal migration) [53] of the observed attenuation of *Arc* induction. This advocates for additional modes of PBDE toxicity, perhaps acting alongside disruption of thyroid hormone signaling. However, we also note that 1 μM 6OH-induced cytotoxicity partially confounds interpretation of this attempted T3 rescue, as reduction of overall cell viability may obscure potential reversal of the effects of 6OH exposure by T3. To explore alternate modes of PBDE toxicity within a context of the relation of PBDE exposure to NDDs, we screened 15 NDD candidate genes, many of which are epigenetic regulators (Table 1), for changes in mRNA level. Subsequently, we found that, while exposure to the parent compound and its other hydroxylated metabolites significantly impacted only a few of these targets, 6OH dysregulated mRNA levels of many of the screened genes. These included *Shank3*, which encodes an important synaptic protein, supporting our inference related to detrimental synaptic effects of persistent 6OH exposure.

Of the genes dysregulated by 6OH exposure, a smaller subset additionally exhibited altered levels of pre-mRNA, indicating a direct effect of 6OH on transcription of these genes. Surprisingly, each of the genes whose pre-mRNA was found to be significantly dysregulated encode BAF chromatin remodeling complex subunits or interacting proteins (*Adnp*, *Arid1b*, *Smarcc2*). One of these genes, *Smarcc2*, encodes a critical core subunit known to contribute to overall BAF complex stability and composition, BAF170. Protein levels of BAF170 were subsequently found to be dysregulated by 6OH exposure, though in a complex oscillatory manner that remains to be fully characterized. It may be the case that as lack of BAF170, but not overabundance, is detrimental for neurons (as we have shown in Fig. 8), the cells are responding to repeated periods of lack of sufficient BAF170 by upregulating *Smarcc2*, while protein levels vary due to post-translational regulation.

BAF170 is a striking candidate whose dysregulation may contribute to the observed effects of 6OH toxicity, owing to its critical contribution to BAF complex function. It has been shown to regulate the stability of BAF chromatin remodeling complexes by acting as a scaffolding subunit and preventing degradation of other BAF proteins. Our results support this notion and demonstrate a reduction of protein levels of several BAF subunits upon BAF170-KD (Fig. 8G), an effect previously only seen with removal of both BAF170 and another interchangeable complex subunit, BAF155 [65]. This may indicate that BAF170 plays a larger role in stabilizing BAF complexes or specific subunits during the periods of neuronal maturation investigated in this study. This stabilizing aspect of BAF170 function, making it critical for BAF complex function as a whole, is intriguing as BAF complexes have well known roles in neuronal differentiation and maturation [60], and are thought to contribute to regulating activity-induced gene transcription [74–76]. As such, periods of 6OH induced lack of BAF170 during early stages where differentiation and maturation are taking place in our cultures may compromise the functional maturation of cells, leading to the observed decrease in *Arc* expression when assayed at later time-points.

Accordingly, we have shown that reduction of BAF170 levels by RNAi, but not its overexpression, negatively impacts neuronal maturation using *Arc* induction as a readout, recapitulating the effect of exposure to 0.5 μM 6OH following Bic + 4AP treatment. The lack of effect seen after BAF170 depletion on extrasynaptically induced *Arc* (TTX-PMA protocol), as was the case with lower doses of 6OH in Figure 3C, indicates that BAF170-containing BAF complexes may not be necessary for nuclear regulation of activity-induced *Arc* transcription, but most likely contribute to synapse maturation and function via transcription regulation of other genes. These findings are consistent with recent evidence supporting the idea that BAF complexes are crucial for synapse formation and maturation during early neuronal development [75]. The same study implicated a role of Brg1, a core BAF complex ATPase, in activity-induced gene transcription 6 h after onset of activity. However, as our data indicate, BAF170-containing BAF complexes are not specifically required for *Arc* induction at early time points if activity is induced extra-synaptically, likely owing to it[CVOAPS]s poised transcriptional status [43]. This lack of effect following BAF170 depletion and TTX + PMA treatment may be gene-specific as various IEGs are regulated in differing manners [43, 52, 76].

While our current work potentially opens new avenues to explore the effects imposed by hydroxylated BDE-47 metabolites on epigenetic regulation during neuronal maturation and activity-induced gene expression, there are limitations to our study. Although we have demonstrated that exposure to 6OH has a strong effect on BAF170 expression, from the pre-mRNA to protein level, the effect is of a complex nature, whose mechanistic basis we do not yet fully understand. Consistant with our findings, the multiphasic expression pattern of BAF170 has been previously demonstrated during corticogenesis in mice [66] and in H1 embryonic stem cells [44], but further investigation will be required to fully characterize the expression profile of BAF170 and regulatory mechanisms that shape it. Additionally, like much PBDE research, we have investigated the effects of exposure to a single toxin at a time, while the developing human nervous system is realistically exposed to complex mixtures of pollutants. Some studies have been completed demonstrating effects of coexposures to environmental pollutants both *in vitro* and *in vivo* using combinations of PBDE congeners, other toxins including polychlorinated biphenyls (PCBs), and commercially available mixtures such as DE-71 [77–80]. More such studies will be needed to address the effects resulting from environmentally relevant mixtures of compounds, especially with concern to their metabolites.

Finally, our results show that nanomolar range exposures to the BDE-47 hydroxylated metabolite 6OH strongly impact several aspects of neuronal maturation and function and that these effects are likely, at least in part, mediated by disruption of BAF chromatin remodeling complexes via dysregulation of BAF170 expression. Going forward, it will be important to fully characterize the regulatory mechanisms governing the intricate expression of BAF170 to discern precisely how the observed PBDE-induced dysregulation is produced. Additional investigation of the underlying mechanisms that mediate the neurotoxic effects of PBDE at the level of epigenetic regulation may lead to a better understanding of how exposure to these compounds leads to developmental complications and NDDs as well as to further characterization of fundamental neuroepigenetic processes.

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Conflict of interest statement. None declared.

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