



OPEN Sex-specific effects of prenatal bisphenol A exposure on transcriptome-interactome profiles of autism candidate genes in neural stem cells from offspring hippocampus

Kasidit Kasitipradit¹, Surangrat Thongkorn², Songphon Kanlayaprasit³, Thanit Saeliw³, Pattanachat Lertpeerapan¹, Pawinee Panjabud¹, Depicha Jindatip^{3,4}, Valerie W. Hu⁵, Takako Kikkawa⁶, Noriko Osumi⁶ & Tewarit Sarachana³✉

Bisphenol A (BPA), an endocrine-disrupting chemical, is increasingly linked to the pathogenesis of autism spectrum disorder (ASD). This study investigates the effects of prenatal BPA exposure on neural stem cells (NSCs) from the hippocampi of rat offspring, a brain region critical for neurodevelopment and implicated in ASD. Pregnant rats were administered with BPA or vehicle control once daily via oral gavage from gestational day 1 until parturition. NSCs were isolated from the offspring's hippocampi on postnatal day 1, and RNA sequencing was performed to examine transcriptomic alterations. Differentially expressed genes (DEGs) were identified through RNA-seq and further analyzed using Ingenuity Pathway Analysis (IPA) to explore disrupted pathways. In addition, *in vitro* proliferation assays were conducted, utilizing immunofluorescence staining for Sox2, a stem cell marker, and BrdU to quantify proliferating NSCs. Our results revealed that prenatal BPA exposure induced sex-specific alterations in NSC gene expression, with ASD-related genes such as *Atp1a3*, *Nefl*, and *Grin1* being particularly dysregulated in male offspring. Moreover, sex-specific changes in NSC proliferation were observed. The study underscores BPA's potential as an environmental risk factor for ASD, emphasizing the need for further research into its role in sex-specific neurodevelopmental effects.

Keywords Autism spectrum disorder, Bisphenol A, Endocrine-disrupting chemical, Transcriptome, Interactome, Neural stem cells, Hippocampus, Sex difference

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by challenges in social communication and the presence of restrictive and repetitive behaviors. It is crucial to recognize that the spectrum signifies a wide range of challenges and strengths, resulting in a diverse spectrum of individuals with ASD¹. The term spectrum does not imply a linear scale from less autistic to more autistic but rather encompasses variation in areas such as executive function, sensory processing, repetitive behaviors, motor skills, perseverative thinking, social awareness, and verbal/nonverbal communication. In recent years, ASD has gained significant

¹The Ph.D. Program in Clinical Biochemistry and Molecular Medicine, Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok 10330, Thailand. ²Department of Biotechnology and Biomedicine (DTU Bioengineering), Technical University of Denmark, 2800 Kgs. Lyngby, Denmark. ³Chulalongkorn Autism Research and Innovation Center of Excellence (Chula ACE), Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University, 154 Soi Chula 12, Rama 1 Road, Wangmai, Pathumwan, Bangkok 10330, Thailand. ⁴Department of Anatomy, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. ⁵Department of Biochemistry and Molecular Medicine, School of Medicine and Health Sciences, The George Washington University, Washington, DC 20052, USA. ⁶Department of Developmental Neuroscience, United Centers for Advanced Research and Translational Medicine (ART), Tohoku University Graduate School of Medicine, Sendai, Miyagi 980-8577, Japan. ✉email: tewarit.sa@chula.ac.th

attention due to its increasing prevalence and impact on individuals and society. As our understanding of ASD grows, it is clear that we need a multidimensional approach to fully comprehend its complexities. The prevalence of ASD has risen steadily, with current estimates indicating that approximately 1 in 36 children at age 8 years old in the United States are diagnosed with ASD^{2,3}. Moreover, ASD is strongly male-biased, affecting 4 times as many males as females, on average^{4,5}. This trend emphasizes the urgent need for extensive research and effective interventions to support individuals with ASD and their families. In understanding ASD, researchers investigate the complex interplay of genetic and environmental factors. While studies reveal a strong genetic component, the intricacies of these genetic factors remain unclear⁶. Recent research has unveiled an association between ASD and epigenetic regulatory mechanisms^{7,8}, which can modulate gene expression without altering DNA sequences and may be responsive to environmental influences^{9–15}. ASD development is thought to result from a combination of genetic and environmental factors, with estimates indicating a near 50–50% contribution from each¹⁶. Environmental factors, notably prenatal exposure to endocrine-disrupting chemicals (EDCs), have been implicated in ASD risk. EDCs, such as bisphenol A (BPA), structurally mimic hormones, disrupting the endocrine system and competitively binding to receptors, thereby impeding natural hormonal actions. This class of chemicals has been particularly scrutinized in relation to ASD due to critical effects during specific developmental windows¹⁷. The examination of EDCs, including BPA, in prenatal and early postnatal exposures aims to discern their distinct impact on neurodevelopment and unravel specific mechanisms contributing to ASD risk^{18,19,20}. BPA, widely used in daily-life products, has raised health concerns due to its ability to disrupt the endocrine system. The accessibility and consumption of BPA heightens apprehensions, given its recognized neurotoxicity^{21–23}. In response to these concerns, regulatory bodies such as the U.S. Food and Drug Administration (USFDA) and the European Food Safety Authority (EFSA) have established No Observed Adverse Effect Level (NOAEL) and Tolerable Daily Intake (TDI) values for BPA at approximately 5,000 µg/kg BW/day and 0.2 ng/kg BW/day, respectively, reflecting a growing awareness of BPA's potential health impacts across diverse age groups^{24–26}. BPA raises concern due to its systemic distribution within the human body, facilitated by its ability to traverse both the placental barrier and the blood–brain barrier^{27,28}. Human exposure to BPA is widespread, as evidenced by detectable levels in various biological matrices such as urine²⁹, fetal sera³⁰, amniotic fluid³¹, placenta³², umbilical cords³³, colostrum³⁴, and breast milk³⁵. Elevated BPA concentrations have been documented in the blood and urine of individuals diagnosed with ASD, particularly in children. Accumulating evidence, particularly from animal models, suggests potential neurotoxic effects of BPA during early developmental stages, exerting adverse influences on neural development, behavior, cognition, neural function, and hormonal signaling pathways^{22,23,36}. According to a prior investigation conducted by Miyakoda et al. (1999), the transplacental transfer of BPA in pregnant rats revealed detectable concentrations of 7.7 ng/g at 24 h post-administration following an oral dose of 10 mg/kg body weight to pregnant Wistar rats³⁷. This observation aligns with another study wherein analysis of blood samples from pregnant mothers identified BPA concentrations ranging from 0.2 to 9.2 ng/mL (median = 2.3 ng/mL) in fetal plasma³². Recently, BPA exposure raised significant concerns, particularly during prenatal and early childhood stages, as research suggests it might impact neurodevelopment^{38–41}. Numerous studies have hinted at a possible connection between BPA exposure and altered brain development, prompting questions about its role in ASD^{23,42–44}. Understanding ASD remains challenging due to its complex neurodevelopmental nature and the absence of precise causative factors. Despite extensive research, the exact origins of the disorder remain elusive. In our study, we hypothesize that BPA might trigger early brain malfunction, suggesting a potential link to ASD. This underscores the importance of employing neural stem/progenitor cells (NSCs) as a model in our investigation. By studying the responses of these cells to BPA, we aim to elucidate the specific cellular characteristics affected. NSCs, being the source of brain cells, serve as an ideal representation for developmental studies, enabling us to potentially trace each cell within the neural lineage. Recent studies from our team have linked gestational exposure to BPA with ASD. Our recent investigations have revealed that maternal exposure to BPA during pregnancy disrupts the transcriptome and interactome profiles of genes in the hippocampus and prefrontal cortex of offspring, exhibiting sex-dependent effects^{38,39}. The identified BPA-responsive genes are linked to ASD and related neurological functions, specifically impacting processes such as neuronal cell proliferation, neurogenesis, neuritogenesis, synaptogenesis, and overall brain development^{39,40,42}. Concurrent research has demonstrated BPA's influence on neocortical development by expediting neuronal differentiation and migration^{45,20}. Furthermore, BPA exhibits neurotoxicity and inhibitory effects on postnatal neurogenesis in the hippocampal dentate gyrus^{44,46}. Despite the increasing clarity on BPA-induced alterations in gene expression and neurological functions associated with ASD in a sex-dependent manner, the specific sex differences in the prenatal exposure effects on the transcriptome-interactome profiles of NSCs in the offspring's brain remain to be elucidated.

In this study, we therefore sought to investigate the sex-specific effects of prenatal BPA exposure on the transcriptome-interactome profiles of NSCs residing in the developing hippocampus of the offspring. First, we performed an RNA-seq transcriptome profiling analysis of NSCs isolated from the hippocampus of neonatal rat pups prenatally exposed to BPA or vehicle control. Genes that were differentially expressed in the BPA treatment group compared to the control group were determined. The lists of differentially expressed genes (DEGs) in the BPA group when both sexes of pups were combined into one group and when male and female pups were analyzed separately were compared with ASD candidate genes from two independent ASD databases to determine whether BPA-responsive genes were significantly associated with ASD candidate genes. Biological functions, pathways, and interactome networks associated with BPA-responsive genes were also predicted using Ingenuity Pathway Analysis (IPA) software. Moreover, we obtained published RNA-seq data of fibroblasts, induced pluripotent stem cells (iPSCs), iPSC-derived neural progenitor cells, and iPSC-derived neurons from ASD cases and typically developing people and reanalyzed them to determine DEGs in these cells. Hypergeometric distribution analysis was then performed to assess the association between the lists of BPA-responsive genes from our study and DEGs in these ASD cells. Significant BPA-responsive genes that are ASD candidate genes were selected for

further confirmation by qRT-PCR analysis. Neurosphere assays were performed using NSCs from the BPA treatment and the control groups. In conjunction with the neurosphere assay, we conducted an additional in vitro proliferation assay to evaluate the influence of prenatal BPA exposure on NSC proliferation. The correlation between the expression of selected ASD candidate genes and the size of neurospheres was then conducted to determine whether these genes may be involved in the proliferation of NSCs.

Results

Prenatal BPA exposure alters the transcriptome profiles of NSCs

To identify the effects of prenatal BPA exposure on the transcriptome profiles of NSCs, a transcriptome profiling analysis was performed using total RNA from the in vitro culture neurosphere isolated from the neonatal rats, both male and female, prenatally exposed, in utero, to the vehicle control or to 5,000 µg/kg-maternal BW of BPA. In vitro cultured neurospheres were immunofluorescent stained with anti-SOX2 antibody, NSC specific marker, to assure that there is a population of stem cells in neurospheres (Fig. 1). According to the Food and Drug Administration (USFDA) and the European Food Safety Authority (EFSA), the dose of BPA that we used to treat rats in this study (5,000 µg/kg-maternal BW/day) is equal to the no-observed-adverse-effect level (NOAEL) for systemic toxicity in humans^{24–26}. We conducted RNA sequencing using the total RNA of NSCs, and the results showed the number of DEGs in the NSCs in response to prenatal BPA exposure. In both sexes groups, a combination of the male and female neonatal rats under the same treatment conditions into one group, as many as 648 genes were significantly differentially expressed in the NSCs of the neonatal rats prenatally exposed to BPA compared with those in the NSCs of the neonatal rats treated with vehicle controls. Additionally, when identifying DEGs in each sex, we found that as many as 71 genes and 27 genes were significantly differentially expressed in the NSCs of the male and female neonatal rats prenatally exposed to BPA, respectively, compared with those in the NSCs of the neonatal rats treated with vehicle controls. (P-value < 0.05 and Q-value < 0.05; Supplementary Table S2, S3). It is claimed by the discovery of altered gene expression upon BPA exposure, as we identify DEGs in the NSCs in response to prenatal BPA exposure, that prenatal exposure, in utero, to BPA, differentially altered the transcriptome profiles of NSCs, both in males and females.

BPA-responsive DEGs in NSCs are associated with ASD

To examine whether these DEGs in response to prenatal exposure to BPA are associated with ASD, we overlapped the list of BPA-responsive DEGs from RNA sequencing analysis with the lists of ASD candidate genes from each of two ASD databases, including the AutismKB and SFARI databases. As many as 169 and 79 genes among BPA-responsive DEGs have been reported as ASD candidate genes in the AutismKB and SFARI databases, respectively (Fig. 2a). When separately identifying the overlapping data in each sex, we discovered that 16 genes from the BPA-responsive DEGs in males and 13 genes, along with 5 genes from the BPA-responsive DEGs in females and 2 genes, were identified as ASD candidate genes in the AutismKB and SFARI databases, respectively (Fig. 2b,c). The total number of DEGs includes genes without assigned gene symbols (e.g., LOC and RNA family identifiers), which are counted in the DEG totals but not displayed in Fig. 2A–C. The lists of BPA-responsive genes associated with ASD, which overlap with the two ASD-related databases referenced in the study, are shown in Supplementary Tables S4–S6. To investigate the correlation between BPA-responsive genes and ASD candidate genes, we conducted hypergeometric distribution analyses. First, we compared the lists of DEGs from both sexes, combining data from male and female neonatal rats with the ASD candidate gene lists. Additionally, we carried out separate analyses for males and females. In these individual analyses, we compared the DEG lists from male and female neonatal rats with the ASD candidate gene lists. The hypergeometric distribution method was employed in all these assessments. Analysis of association P-values, as presented in Supplementary Table S7 (P-value < 0.05), revealed significant enrichment of ASD-related genes within the lists of DEGs in both sexes and in male neonatal rats. This enrichment was observed in data sourced from two ASD databases, namely, AutismKB and SFARI. However, when female pups were analyzed independently, the list of DEGs did

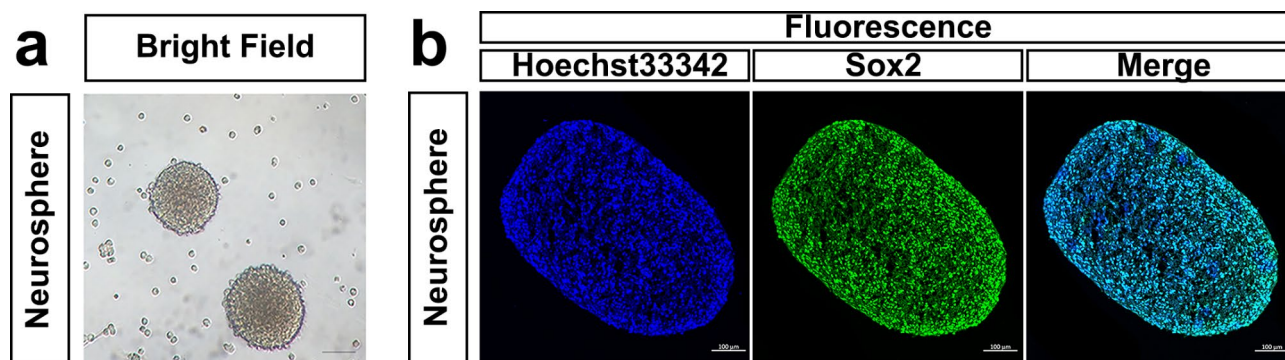


Fig. 1. Characterization of NSCs within the cultured neurospheres. (a) A bright field microscopy image of neurosphere DIV14. (b) The immunofluorescence staining of DIV14 neurospheres. Neurospheres were immunofluorescence stained with anti-Sox2 antibody to further identify neural progenitor cells. ((a) Scale bar = 10 µm; (b) Scale bar = 100 µm).

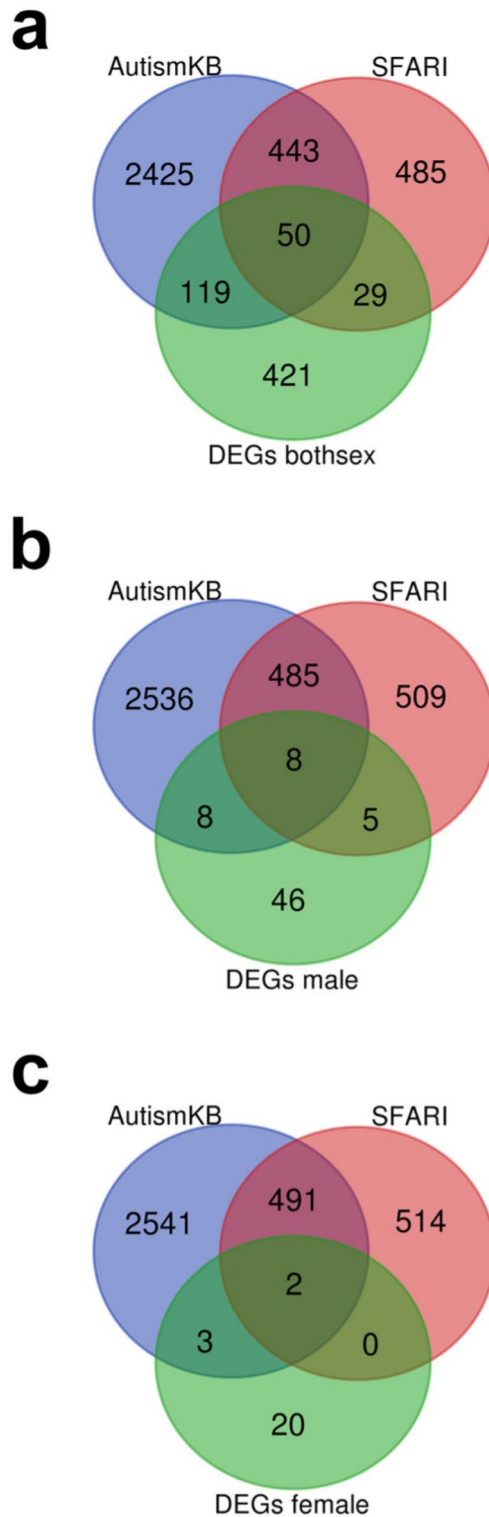


Fig. 2. Venn diagram of BPA-responsive DEGs and ASD candidate genes. **(a)** The list of BPA-responsive genes in the group with both sexes overlapped with the list of ASD candidate genes from each ASD database (the SFARI and AutismKB databases). **(b)** The list of BPA-responsive genes in the male group overlapped with the list of ASD candidate genes from each ASD database (the SFARI and AutismKB databases). **(c)** The list of BPA-responsive genes in the female group overlapped with the list of ASD candidate genes from each ASD database (the SFARI and AutismKB databases).

not demonstrate a significant association with ASD genes. These findings suggest that BPA-responsive DEGs in NSCs are linked to ASD genes. Moreover, DEGs in male NSCs exhibited a more pronounced association with ASD compared to those in female NSCs.

BPA-responsive DEGs in NSCs are involved in biological diseases, especially neurological diseases associated with ASD

To predict biological functions, pathways, and interactome networks associated with BPA-responsive DEGs in the hippocampal NSCs, the lists of DEGs from RNA sequencing analysis were analyzed using Ingenuity Pathway Analysis (IPA) software. The interactome/regulatory network of DEGs in NSCs generated by IPA software showed a relationship to several neurological diseases/disorders and functions that are impacted in ASD, such as cognitive impairment, memory deficits, neurodevelopmental disorder with poor language and loss of hand skills, motor dysfunction, memory, and etc. (Fig. 3). After analyzing the lists of BPA-responsive DEGs through the use of IPA software, the program unveiled associations between biological diseases and disorders and these lists, both when considering both sexes together and separately for males and females. Notably affected diseases and functions following prenatal BPA exposure included "neurological diseases," "psychological disorders," "organismal injury and abnormalities," "cancer," "dermatological diseases and conditions," "skeletal and muscular disorders," "metabolic disease," and "infectious disease" (Supplementary Table S8). Particularly intriguing, within this group, "neurological diseases" and "psychological disorders," which are presented in top diseases and bio functions and linked to ASD, displayed significant alterations as determined by Fisher's exact test (P -value < 0.05). Furthermore, upon analysis of the BPA-responsive differentially expressed coding sequences (CDS), neurological diseases and psychological disorders exhibited significant changes, consistent with the predictions derived from the DEG lists (P -value < 0.05 , Supplementary Table S9). Moreover, we also predicted the molecular and cellular functions using the lists of DEGs and the lists of differentially expressed CDS (Supplementary Table S10, Supplementary Table S11). Interestingly, after we used IPA software to predict the neurological diseases/disorders using the lists of BPA-responsive DEGs from the NSCs, it revealed that several neurological diseases were associated with these DEGs, such as autism, cognitive impairment, mental retardation, movement disorder, and pervasive developmental disorder (Supplementary Table S12). It is very interesting that these lists of neurological diseases/functions were significantly (P -value < 0.05) associated in the sex difference manner by significantly associated in both sexes, and in males rather than in females. We also used IPA software to predict the canonical pathway associated with DEGs from the RNA-seq and as shown in Supplementary Table S13, "GABA receptor signaling," "glutamate receptor signaling," "synaptic long-term potentiation," and "nNOS signaling pathway," all of which have been reported to be associated with ASD^{47–49}, were found to be significantly associated with DEGs in NSCs with the P -value < 0.05 . Among the canonical pathways predicted by IPA software, GABA receptor signaling was present in both sexes and male and female NSCs; glutamate receptor signaling and neuronal nitric oxide (nNOS) signaling in neurons were present in both sexes and male NSCs.

DEGs from multiple transcriptomic studies revealed an association with ASD candidate genes based on the integration of data from multiple studies

To determine whether genes that were differentially expressed in iPSC-derived ASD patients and identified by independent studies were also associated with BPA-responsive genes from RNA-seq data, transcriptome profiling data from iPSC-derived cells from control and ASD patients were obtained from three independent transcriptomic studies; the data were previously deposited in the NCBI Gene Expression Omnibus (GEO) DataSets database (<https://www.ncbi.nlm.nih.gov/gds/>). The microarray data from each GEO repository were uploaded to the Multiple Experiment Viewer program (MeV) for statistical analysis. The GEO accession numbers used in this study were [GEO: GSE65106], [GEO: GSE67528], and [GEO: GSE124308]. The details of each study, including the title, sample size, and sample type, are shown in Supplementary Table S14. We then overlapped these DEG lists from each study with the ASD candidate genes from two different ASD databases: SFARI (<https://gene.sfari.org/>) and AutismKB (<http://autismkb.cbi.pku.edu.cn/>). We further performed hypergeometric distribution analyses to determine whether the lists of DEGs from each study were associated with the BPA-responsive genes from our study. These results showed that DEGs in iPSC-derived cells from ASD patients were significantly (P -value < 0.05) associated with the ASD candidate genes, especially in both sexes and in male NSCs. The association analyses between the DEGs from our study and the DEGs in ASD are shown in Supplementary Table S15.

Quantitative RT-PCR analysis of selected BPA-responsive DEGs in NSCs

To further examine whether prenatal BPA exposure causes the dysregulation of genes in NSCs, five DEGs (i.e., *Atp1a3*, *Nefl*, *L1cam*, *Grin1*, and *Gabbr1*) identified by RNA-seq analysis and reported as ASD candidate genes, were selected for further confirmation by qRT-PCR analysis in another set of NSC samples (Fig. 4). ATPase Na⁺/K⁺ transporting subunit alpha-3 (*Atp1a3*), neurofilament light (*Nefl*), L1 cell adhesion molecule (*L1cam*), glutamate ionotropic receptor NMDA type subunit 1 (*Grin1*), and GABA(A) receptor subunit beta-1 (*Gabbr1*), were predicted to be linked to neurological disorders and developmental disorders, particularly autism, and have been identified as ASD candidate genes. We found that *Atp1a3*, *Grin1*, and *Gabbr1* were significantly upregulated in the NSCs of rats prenatally exposed to BPA when both males and females were combined (P -value < 0.05 , Fig. 4a). When separately analyzed in males, the expression levels of all genes except *L1cam* were significantly increased (P -value < 0.05 , Fig. 4b). However, in females, the expression level of all selected genes tended to increase after being prenatally exposed to BPA, although the difference was not statistically significant (P -value < 0.05 , Fig. 4c). These results indicate that prenatal BPA exposure causes the dysregulation of genes associated with ASD in hippocampal NSCs in the sex-dependent manner.

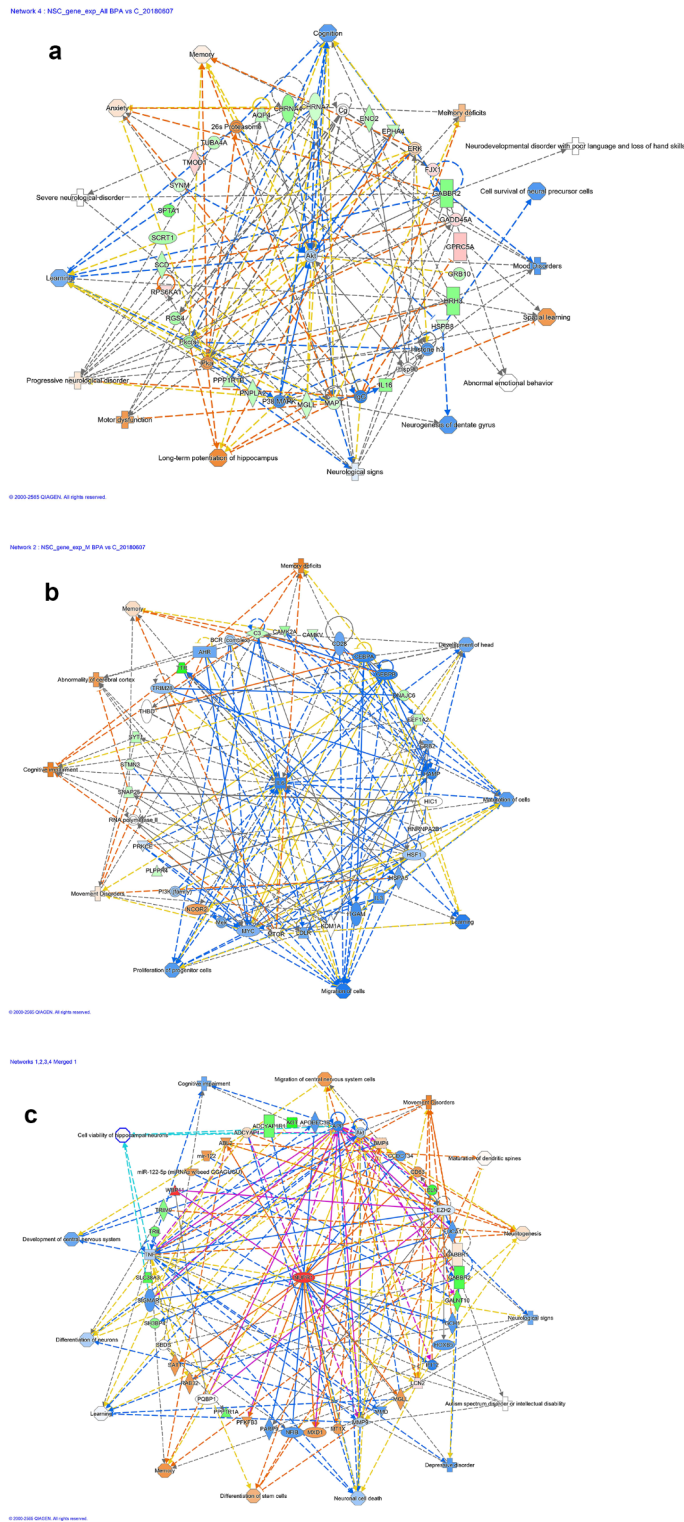


Fig. 3. The regulatory network of DEGs in NSCs is related to neurological diseases/disorders and functions that are impacted in ASDs. The gene regulatory network of both sexes (a) males (b) and females (c) was predicted by IPA software using the list of DEGs from RNA-seq, and the IPA showed that these genes are related to neurological diseases and functions that are impacted in ASD.

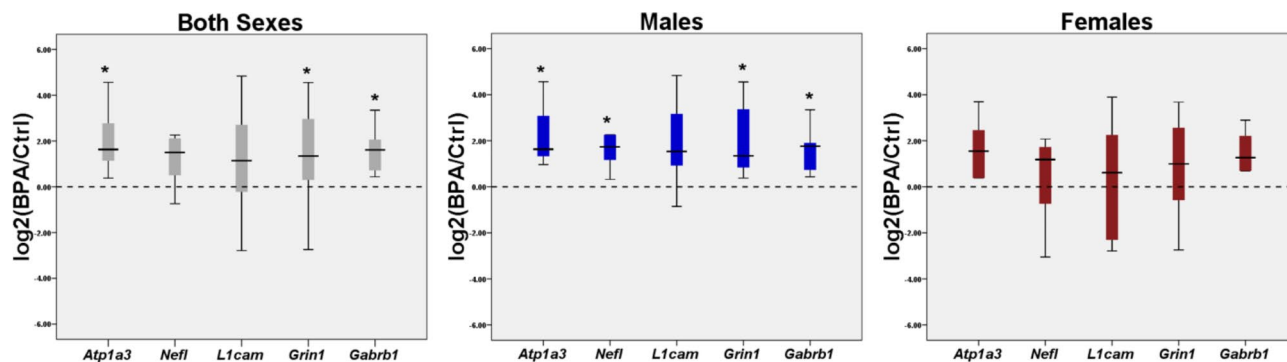


Fig. 4. The box plot of ASD-related gene expression in NSCs. The expression levels of *Atp1a3*, *Nefl*, *L1cam*, *Grin1*, and *Gabrb1* were determined in both sexes and separately in males and females. The qRT-PCR analyses revealed that *Atp1a3*, *Grin1*, and *Gabrb1* were significantly upregulated in the NSCs of both sexes that were prenatally exposed to BPA. All genes except *L1cam* were significantly upregulated in the NSCs in the male group. The expression level of every gene tended to increase in the NSCs in the female group but was not statistically significant. * P-value < 0.05, paired *t*-test.

Correlation between BPA-responsive DEGs and size of neurospheres

To examine the effects of prenatal BPA exposure on NSC characteristics, we were interested in the cell proliferation of NSCs, and hence, the neurosphere assay was performed by culturing NSCs in vitro for 1–2 weeks. The analysis was conducted in accordance with a script written in ImageJ macro, which was slightly modified from Ivanov et al. (2014)^{50,2} (n = 709 spheres) and DIV14 was 18,746.14 μm^2 (n = 620 spheres) and the mean area of neurospheres in the BPA treated group at DIV7 was 14,072.53 μm^2 (n = 1,003 spheres) and DIV14 was 29,042.66 μm^2 (n = 935 spheres). In males, the mean area of neurospheres in the control group at DIV7 was 9,709.90 μm^2 (n = 291 spheres) and DIV14 was 11,554.54 μm^2 (n = 231 spheres), and the mean area of neurospheres in the BPA treated group at DIV7 was 12,867.85 μm^2 (n = 354 spheres) and DIV14 was 24,768.97 μm^2 (n = 259 spheres). In females, the mean area of neurospheres in the control group at DIV7 was 9,441.55 μm^2 (n = 418 spheres) and DIV14 was 23,016.74 μm^2 (n = 389 spheres) and the mean area of neurospheres in the BPA treated group at DIV7 was 14,729.62 μm^2 (n = 649 spheres) and DIV14 was 30,680.07 μm^2 (n = 676 spheres). Analysis of variance (ANOVA) using Tukey's post hoc showed that the mean area of neurospheres in the BPA-treated group was significantly larger than the mean area of the neurospheres in the control group on both DIV7 and DIV14 (P-value < 0.001), as shown in (Fig. 5).

To further examine that the changes in the expression levels of genes in response to BPA, which we selected to confirm by performing qRT-PCR, were possibly associated with the cell proliferation properties, we then performed a correlation analysis to show a correspondence between the expression levels of selected BPA-responsive DEGs and the area of the neurospheres. Here we selected 5 DEGs, which we confirmed expression level by qRT-PCR, to perform correlation analysis. A correlation between these 5 DEGs (*Gabrb1*, *Grin1*, *L1cam*, *Nefl*, and *Atp1a3*) and the area of neurospheres in both sexes group showed a moderate positive correlation in both sexes group with R coefficient = 0.31, 0.34, 0.35, 0.37, and 0.35, respectively. Interestingly, among the five differentially expressed genes (all genes except *Gabrb1*), four exhibited a stronger positive correlation with the area when specifically analyzed in males, in comparison to the combined data from both sexes. ($R_{\text{Gabrb1}} = 0.38$, $R_{\text{Grin1}} = 0.54$, $R_{\text{L1cam}} = 0.58$, $R_{\text{Nefl}} = 0.54$, $R_{\text{Atp1a3}} = 0.53$). In females, all DEGs showed a low correlation with the area ($R_{\text{Gabrb1}} = 0.15$, $R_{\text{Grin1}} = 0.14$, $R_{\text{L1cam}} = 0.16$, $R_{\text{Nefl}} = 0.07$, $R_{\text{Atp1a3}} = 0.1$). As shown in Fig. 6, changes in the expression levels of selected BPA-responsive DEGs exhibited a sex-difference pattern with a higher correlation in males than in females.

Furthermore, to ensure that our NSC transcriptome remained stable following in vitro culture, we conducted a correlation analysis comparing our NSC transcriptome with transcriptome profiles at various stages of neural development, including in vivo neural stem cells (NSCs), basal progenitors (BPs), and newborn neurons (NBN), as obtained from GSE134688. The results consistently demonstrated a high correlation between our NSC transcriptome and the transcriptome profiles of postnatal (PN) NSCs, both when considering both sexes and when analyzing males and females separately in both the control and BPA-treated groups. This observation strongly suggests that our NSC transcriptome remained unchanged following in vitro culture (Fig. 7).

In vitro proliferation

In conjunction with the neurosphere assay, we conducted an additional in vitro proliferation assay to evaluate the influence of prenatal BPA exposure on NSC proliferation. This assay involved immunofluorescent staining of cultured neurospheres using primary antibodies, including anti-Sox2 and anti-BrdU, followed by counterstaining with Hoechst33342 (Fig. 8). The results demonstrated a significant increase in the total number of cells (Hoechst+), accompanied by a notable reduction in the total number of proliferating NSCs (Sox2+ BrdU+) in both sexes following prenatal BPA exposure. Subsequent analysis of each sex revealed a marked elevation in the proportion of proliferating NSCs relative to the total NSC population (Sox2+ Hoechst+) in males. In females, there was a significant increase in area, total cell count, and total NSCs; however, a concurrent decrease was observed in the density of proliferating NSCs and the percentage of proliferating NSCs per total cell. The

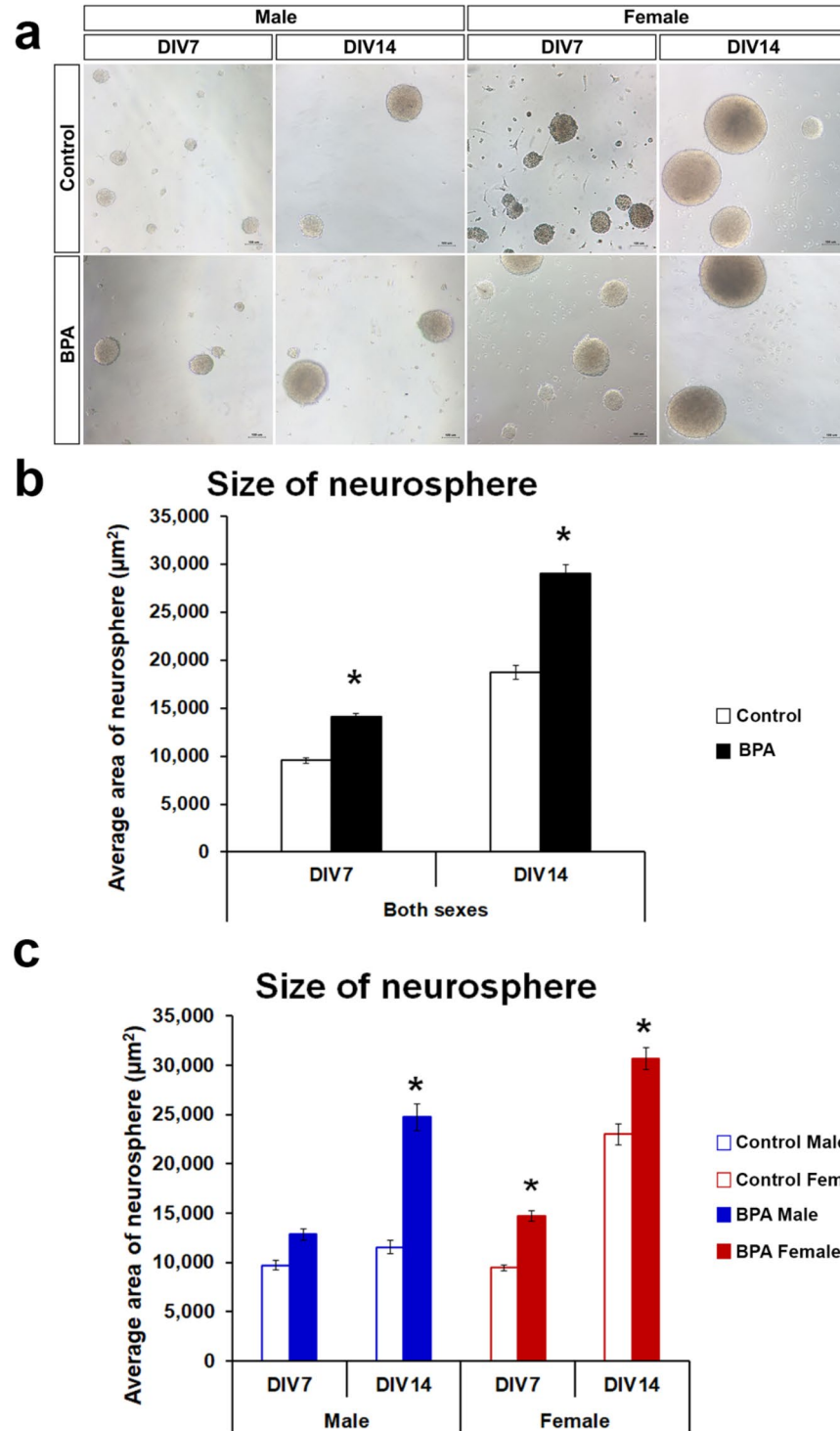


Fig. 5. Neurosphere assays. (a) NSCs of control and BPA treatment pups, both males and females, were cultured in vitro for 7 days and 14 days to form neurospheres. (b) Quantitative analysis of the mean size of neurospheres, both sexes, derived from control or BPA treatment. The mean size of neurospheres derived from BPA treatment was significantly greater than that of the control group. (c) Quantitative analysis of the mean size of neurospheres, separately male and female, derived from control or BPA treatment. The mean size of neurospheres derived from BPA treatment was significantly greater than that from the control group, both in males and females. ((a) Scale bar = 100µm)

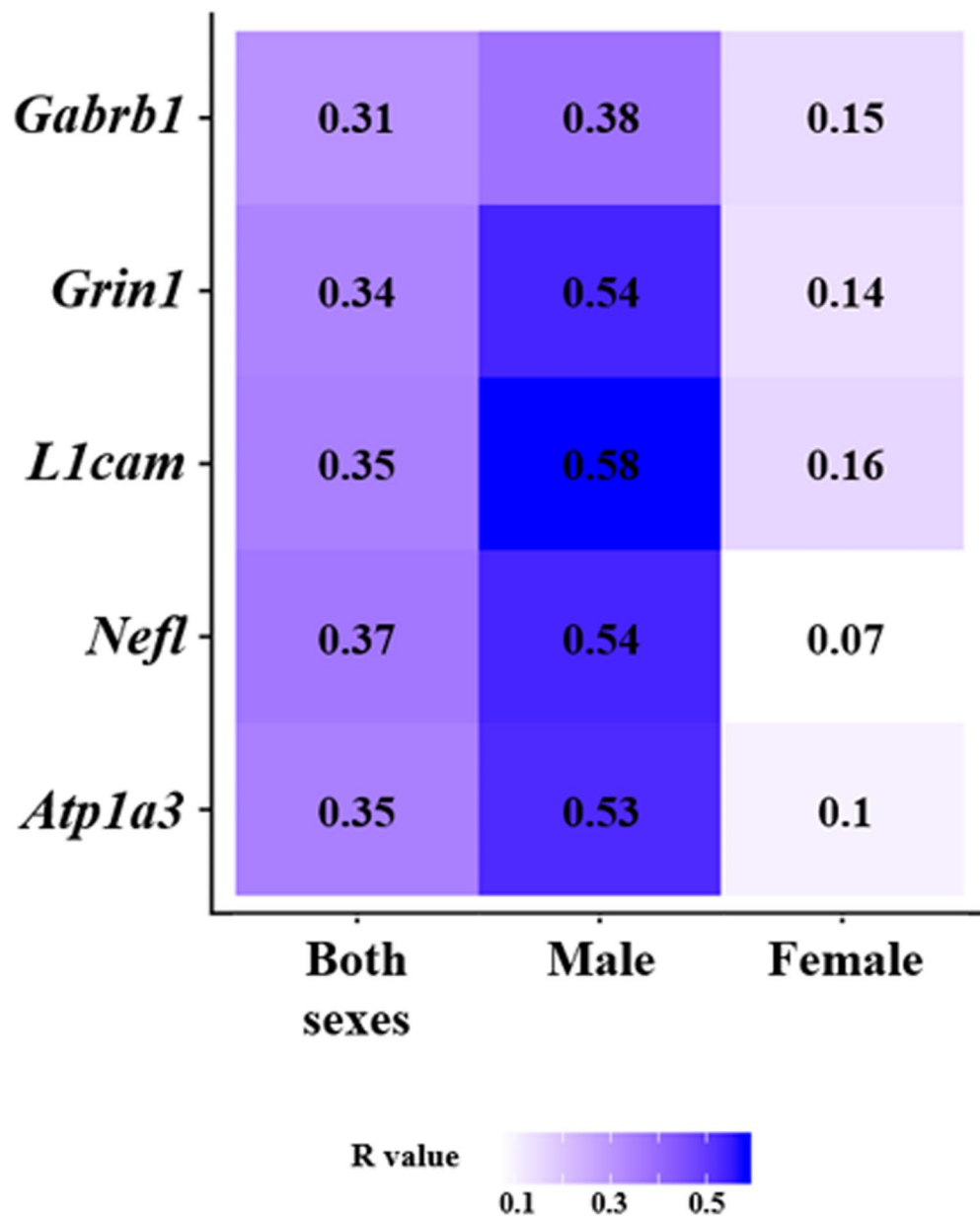


Fig. 6. A correlation between the expression levels of selected BPA-responsive DEGs and the area of the neurospheres.

results indicate a significant reduction in total proliferating NSCs in both sexes after prenatal BPA exposure. Sex-specific alterations in the proportion of proliferating NSCs were observed (P -value < 0.05 , Fig. 9). These findings, the in vitro proliferation assay conducted in conjunction with the neurosphere assay, underscore the sex-specific responses of NSCs to prenatal BPA exposure, providing valuable insights into the intricate dynamics of NSC proliferation influenced by prenatal exposure to BPA.

Discussion

According to the accumulating evidence both in animal models and in humans, BPA has been reported to accumulate in exposed pregnant mothers and can also transfer across the placental barrier to the fetuses. Here, we report on RNA-seq, Ingenuity Pathway Analysis, hypergeometric distribution analysis, qRT-PCR, neurosphere assay, correlation analysis, and in vitro proliferation assay using hippocampal NSCs from neonatal rat pups prenatally exposed to BPA. RNA-seq data showed the number of DEGs, and differentially expressed CDS. This tends to show sex differences in response to prenatal BPA exposure and is consistent with our previous report that prenatal BPA exposure disrupts the transcriptome profile in neonatal hippocampi^{39,40}. Additionally, the trend of the result in this study, male bias towards ASD, was also consistent with the results of many studies^{38–40,51,52}.

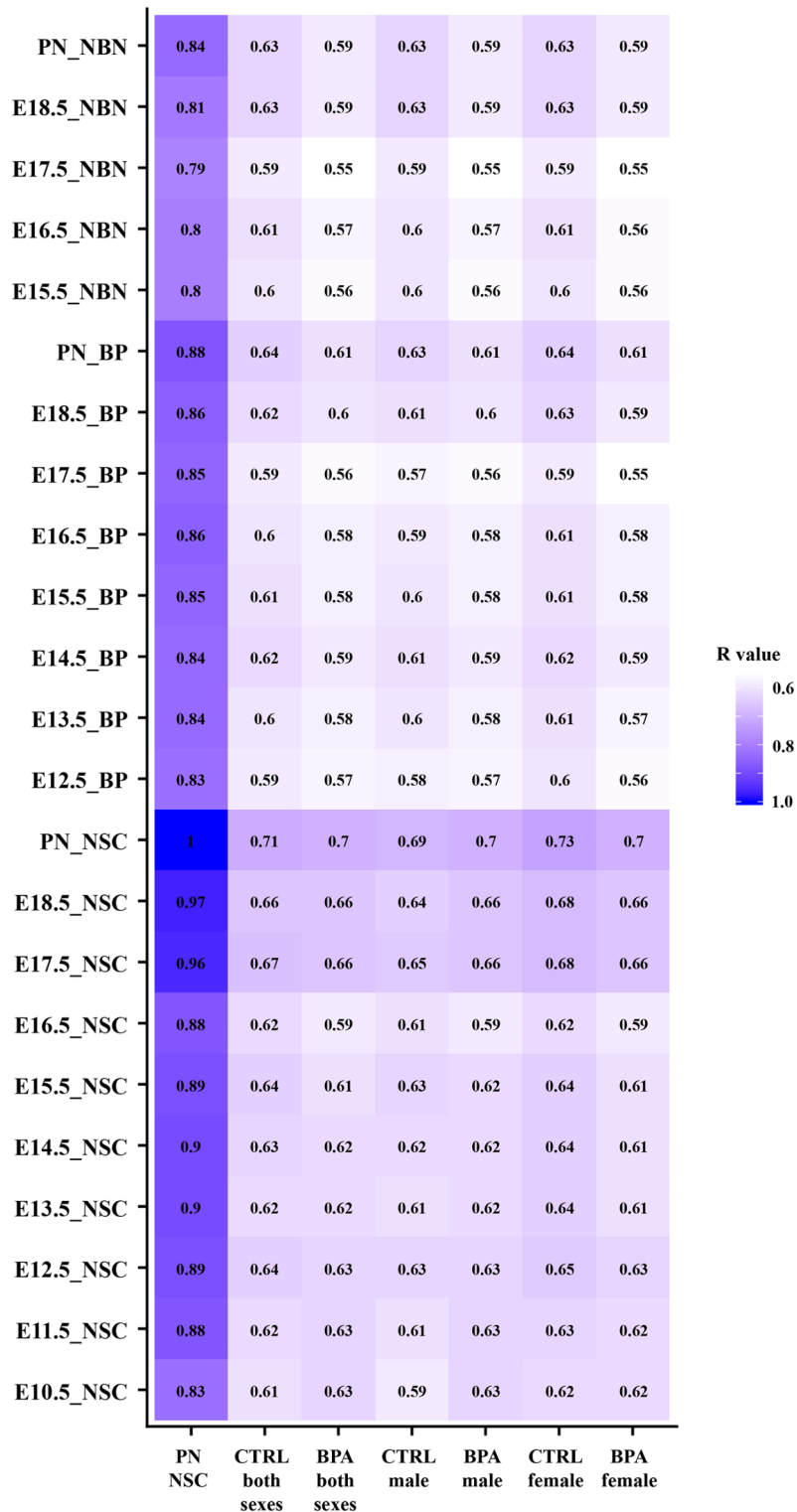


Fig. 7. A heatmap of the correlation between our NSC transcriptome and in vivo NSC, BP, and NBN transcriptome was obtained from GSE134688.

Upon overlapping the lists of DEGs and differentially expressed CDS in response to BPA exposure with ASD candidate genes, we observed an enrichment of genes previously associated with ASD. This pattern was consistent in rat hippocampi, with both DEGs and CDS exhibiting stronger associations with ASD genes in males than females. These findings suggest that prenatal BPA exposure may elevate the risk of ASD. Further analysis using IPA revealed canonical pathways associated with DEGs in both sexes, such as "GABA receptor signaling," "glutamate receptor signaling," "synaptic long-term potentiation," and "nNOS signaling pathway," all

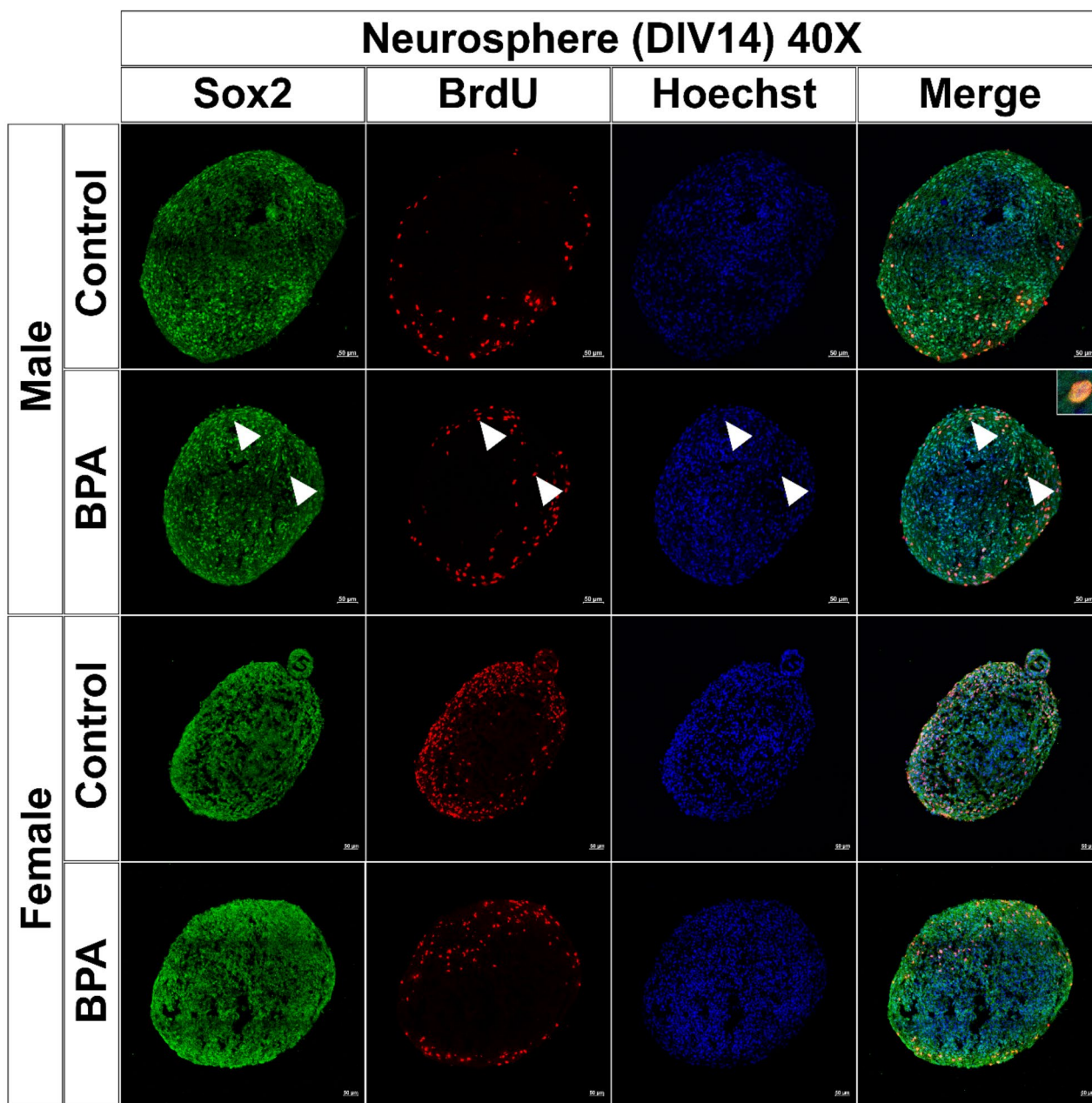


Fig. 8. Proliferating neural stem cells in the cultured neurosphere. The arrowheads label Sox2 + BrdU + cells. The inset demonstrates a Sox2 + BrdU + cell. ((a) Scale bar = 50 μ m).

have been reported to be linked to ASD. Given ASD's neurodevelopmental nature, dysregulation of neurogenesis emerges as a potential key factor in its development, as nonfunctional neurons are produced. Notably, GABA and glutamate receptor signaling pathways have implications in neuronal development, as supported by prior studies. Gascon et al. (2006) demonstrated GABA's role in dendritic development, particularly in postnatally generated olfactory interneurons. Additionally, the nNOS signaling pathway, implicated in various developmental processes, including neuronal development, precursor proliferation, and differentiation, has been associated with negative impacts on neurogenesis in the dentate gyrus in some studies^{53,54}.

In addition to canonical pathways, neurological disorders reflected a consistent trend in DEGs across both sexes, with males exhibiting stronger associations with ASD. "Autism," "Mental retardation," "PDD-NOS," "Speech and language disorders," and "X-linked mental retardation" were significantly associated with DEGs specifically in males, whereas "cognitive impairment," "movement disorders," and "pervasive developmental disorder" were linked to both males and females. Notably, cognitive and movement disorders are prominent features of ASD. Previous research indicating BPA's impact on cognition in adult mice⁵⁵, with a more pronounced effect in males, aligns with this sex-dependent response. Interactome analysis using IPA revealed interactions

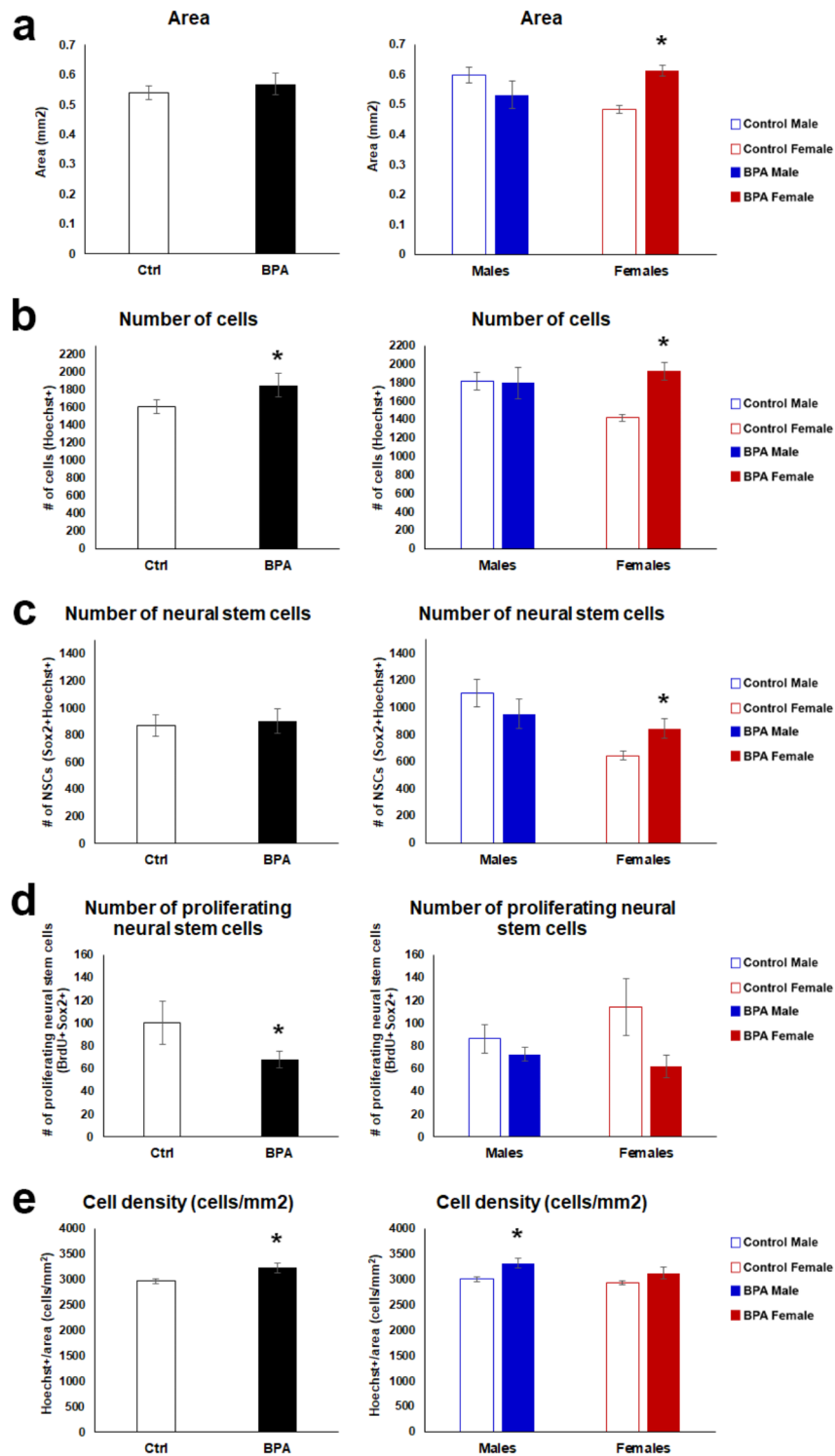


Fig. 9. In vitro neurosphere assay (Day in vitro 14). Quantification of area (a), total cells (b), total neural stem cells (c), total proliferating neural stem cells (d), cell density (e), neural stem cell density (f), proliferating neural stem cells density (g), % proliferating neural stem cells/total neural stem cell (h), % total neural stem cells/total cells (i), and % proliferating neural stem cells/total cells (j) in the cultured neurosphere.

between DEGs and ASD-associated neurological functions. DEGs were predicted to interact with functions such as cognitive impairment, memory, anxiety, motor dysfunction, and neurogenesis of the dentate gyrus, mirroring conditions often found in autistic patients. This suggests that prenatal BPA exposure induces gene expression

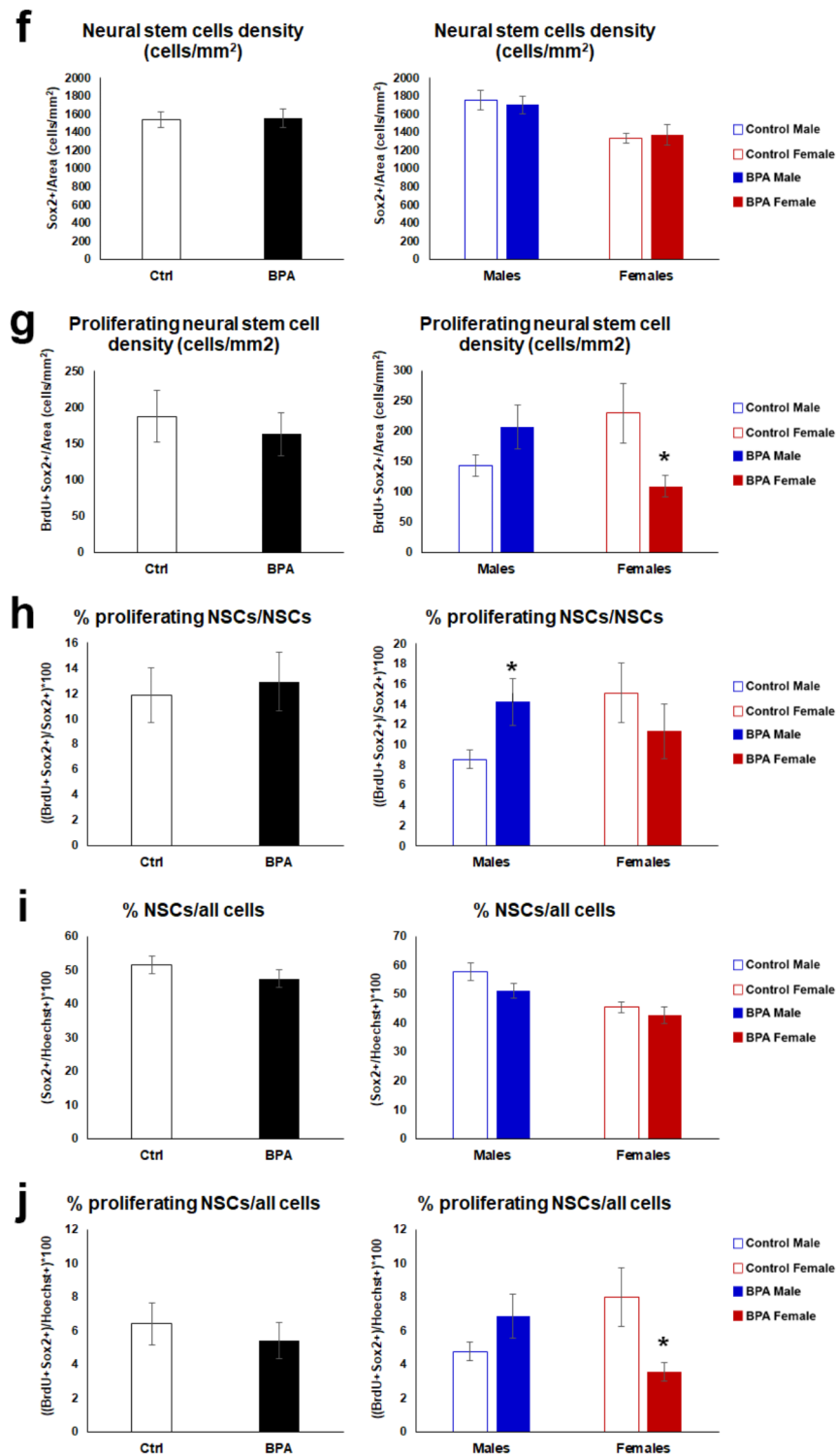


Figure 9. (continued)

changes associated with ASD, emphasizing the relevance of these findings to neurological function and ASD pathogenesis.

To explore the association between BPA, NSCs, and ASD, we identified DEGs using transcriptome profiles of genes from iPSCs-derived cells in typically developing individuals and ASD cases. Employing a t-test with standard Bonferroni correction, we reanalyzed the data to identify dysregulated genes. Subsequently, the lists of DEGs from rat NSCs identified through RNA-seq in this study overlapped with those from each transcriptomic study. Hypergeometric distribution analyses revealed an association between genes in NSCs differentially

expressed upon prenatal BPA exposure and ASD, suggesting that BPA-responsive genes were enriched in iPSCs-derived cells in ASD patients.

Quantitative RT-PCR analyses were conducted to delve deeper into the expression levels of five identified DEGs, namely *Atp1a3*, *Nefl*, *L1cam*, *Grin1*, and *Gabrb1*, selected from the overlap between RNA-seq analysis and ASD candidate genes.

Firstly, these five DEGs were chosen due to their overlap with ASD candidate genes. This overlap suggests that they may play significant roles in the molecular mechanisms linking prenatal BPA exposure to ASD-related phenotypes. Additionally, our Ingenuity Pathway Analysis (IPA) further validated these genes by comparing our RNA-seq results with curated databases of known ASD-related genes and pathways. IPA confirmed that these genes are involved in canonical pathways associated with neurodevelopmental disorders, particularly ASD.

Beyond their association with ASD, these genes were selected for their critical roles in neurodevelopment, including key processes in NSC function:

Atp1a3: Essential for maintaining ionic gradients crucial for neural progenitor cell homeostasis, and influences NSC proliferation, differentiation, and migration by regulating membrane potentials and intracellular signaling⁵⁶.

Nefl: Known for its role in neuronal maturation and cytoskeletal integrity, *Nefl* also supports early stages of neurogenesis by providing structural support during neuronal differentiation and maturation^{57–63}.

L1cam: Involved in cell adhesion, axonal guidance, and migration, which are vital not only for mature neurons but also during NSC proliferation, differentiation, and spatial organization of emerging neurons^{64,65}.

Grin1: Encoding an NMDA receptor subunit, *Grin1* is crucial for synaptic plasticity and early neurogenesis, influencing NSC proliferation and fate determination^{66–69}.

Gabrb1: A component of the GABA receptor complex, *Gabrb1* regulates inhibitory neurotransmission and plays a role in early NSC proliferation and differentiation, with GABAergic signaling modulating neural progenitor development^{70,71}.

These genes reflect key aspects of both early-stage neural development and later neuronal functions, underscoring their relevance to studying the impact of prenatal BPA exposure on NSCs and neurogenesis. The convergence of evidence from our RNA-seq data, IPA analysis, and the established roles of these genes in neurodevelopment and ASD supports their selection as focal points for further analysis.

According to previous studies, *Atp1a3*, crucial for ion gradient maintenance, exhibited increased activity in the frontal cortex of autistic patients⁵⁶. *Nefl*, a neurofilament light component, showed downregulation in the cortex and cerebellum^{57,58}, with upregulation implicated in brain development and ASD-like models. *L1cam*, an adhesion molecule, plays diverse roles in neurodevelopment^{64,65}, with increased expression linked to BPA exposure. *Grin1*, encoding NMDA receptors, exhibited upregulation in cerebellar samples from autistic individuals^{66,67}. *Gabrb1*, an inhibitory GABA receptor, is generally downregulated in ASD-related brain regions⁷⁰. In this study, all five DEGs were significantly elevated in NSCs of the BPA-exposed group in both sexes, with males exhibiting stronger significance. Notably, qRT-PCR results showed an increasing trend in females as well, though not statistically significant. This aligns with the interactomics data from IPA, supporting the notion that prenatal BPA exposure induces gene expression changes associated with ASD. Discrepancies between qRT-PCR and RNA-seq results may arise from cohort differences, underscoring the importance of considering methodological variations in interpreting findings. The pooled nature of RNA-seq analysis may introduce false positives or negatives, emphasizing the necessity of validating results through individual sample analyses.

To elucidate the impact of prenatal BPA exposure on NSC proliferation, a neurosphere assay was performed, a valuable in vitro technique for studying NSCs. The findings from the neurosphere assay align with the previously discussed transcriptomic and interactomic data, collectively contributing to a comprehensive understanding of the impact of prenatal BPA exposure on NSCs. The observed significant increase in the mean size of neurospheres in the BPA-exposed group, particularly in females, resonates with the sex-specific alterations in gene expression profiles identified through RNA-seq and interactomic analyses. Notably, the neurosphere growth rate was higher in BPA-exposed males, mirroring the sex-dependent trends observed in the molecular analyses. Furthermore, the neurosphere assay results align with the sex-dependent effects noted in the transcriptomic analyses, emphasizing the importance of considering gender-specific responses to environmental exposures. The larger neurosphere size in females, coupled with the distinct growth rates between males and females in the BPA-exposed group, suggests nuanced sex-dependent effects on NSC proliferation. To explore the potential correlation between gene expression changes in response to BPA and alterations in neurosphere size, a correlation analysis was performed on five selected DEGs (*Atp1a3*, *Nefl*, *L1cam*, *Grin1*, and *Gabrb1*). The heatmap in Fig. 6 illustrates a strong correlation between *Grin1*, *L1cam*, *Nefl*, and *Atp1a3* with neurosphere area, while *Gabrb1* shows a moderate positive correlation, specifically in males. These findings imply a connection between changes in neurosphere size and gene expression levels in response to BPA. Notably, qRT-PCR results confirmed significant upregulation of these DEGs in males, aligning with reported functions related to brain development, NSC proliferation, and neurogenesis, consistent with neurosphere assay outcomes. In females, although the correlation is lower, it corresponds to non-significant changes in qRT-PCR results. Additionally, to ensure the stability of our in vitro cultured NSC transcriptome, we conducted a correlation analysis comparing it to various developmental stages' transcriptome profiles, including in vivo NSCs, basal progenitors (BP), and newborn neurons (NBN) from GSE134688. Results demonstrated a robust correlation, reinforcing the consistency of our NSC transcriptome after in vitro culture validating the reliability of our experimental approach.

In vitro proliferation assays provide additional insight into the mechanisms underlying BPA-induced changes in NSC behavior. As shown in Fig. 5, we observed a marked increase in neurosphere size at both DIV7 and DIV14 in BPA-treated groups across both sexes. This initially suggests enhanced NSC proliferation or cell survival. However, a deeper analysis in Fig. 9 revealed that, despite the larger neurosphere size, the density of proliferating NSCs was actually reduced in both sexes. This reduction was particularly evident in females, where

a significant decrease in the proportion of proliferating cells relative to the total NSC population was observed. These findings suggest that while BPA exposure increases the overall neurosphere area—potentially through mechanisms that promote cell survival or differentiation—it does not necessarily enhance active proliferation within the neurospheres. Furthermore, the sex-specific patterns observed, with males showing an elevated proportion of proliferating NSCs and females exhibiting increased neurosphere size but decreased proliferative density, highlight differential BPA effects on NSC homeostasis. This complex response underscores the intricate ways in which prenatal BPA exposure can influence NSC dynamics and the developmental trajectory of neural tissues.

IPA analysis of DEGs in response to BPA exposure revealed differential expression of genes involved in neurogenesis, cellular stress response, and cell proliferation pathways, further elucidating the molecular mechanisms behind altered NSC behavior. Key genes such as *Chrna7*, *Chrna4*, *Aqp4*, and *Rpa6ka1* are associated with neurogenesis and cell growth regulation, suggesting their potential roles in modulating NSC dynamics in the hippocampus^{72–74}. In male offspring, additional sex-specific genes—including *Myc*, *Mtor*, *Cebpb*, and *Il6*—were differentially expressed, indicating that BPA exposure may particularly affect cell proliferation pathways in males through these regulators.

While the genes validated by qRT-PCR (*Atp1a3*, *Nefl*, *L1cam*, *Grin1*, and *Gabrb1*) are not direct regulators of cell cycle progression, they play critical roles in maintaining NSC niche structure, signaling, and cellular integrity. This may indirectly facilitate NSC proliferation, as reflected in the increased neurosphere size observed in BPA-exposed groups. This combination of neurodevelopmental and stress-response gene dysregulation likely creates an environment conducive to enhanced NSC proliferation following prenatal BPA exposure, aligning with our observed increase in neurosphere size.

Interestingly, the increased neurosphere size observed in females compared to males may reflect an additional layer of response, potentially driven by estrogen pathways⁷⁵. Despite the stronger transcriptomic changes in males, females exhibited a functional increase in NSC proliferation, possibly through non-genomic mechanisms or pathways not captured by RNA-seq analysis⁷⁶. This underscores the complex interplay of genetic, hormonal, and epigenetic factors influencing NSC behavior and, potentially, ASD risk.

In contrast, Singh et al. reported a decrease in neurosphere size following BPA exposure, which appears contradictory to our findings. This discrepancy may be explained by several key differences in experimental design: Singh et al. used a high, acute dose of BPA (100 μ M) for a short duration (24 h) in vitro, whereas our study focused on in vivo prenatal exposure with NSCs derived from BPA-exposed rats, without additional BPA added during in vitro culturing. This distinction—prenatal exposure in vivo versus acute in vitro treatment—likely contributes to the divergent outcomes in NSC behavior, as in vivo exposure may lead to more prolonged and systemic hormonal and developmental effects.

Epigenetic modifications offer another potential explanation for these differences. BPA, as an endocrine disruptor, influences gene expression through epigenetic changes, including DNA methylation, histone modification, and non-coding RNA regulation^{9,10,7,8}. These modifications can have lasting effects on neurogenesis, especially during critical prenatal developmental windows. For example, BPA exposure has been shown to alter the methylation status of genes involved in neurodevelopment and NSC proliferation, potentially resulting in differential neurosphere growth patterns⁷⁷. In females, the interaction between BPA and estrogen receptors might enhance epigenetic modifications that promote NSC proliferation, contributing to the larger neurosphere size observed in our study.

In this study, we used a relatively high dose of BPA (5 mg/kg/day), which corresponds to the systemic NOAEL in adult rats, to examine the effects of prenatal exposure on hippocampal NSCs and gene expression associated with ASD. This choice of dosage was based on previous studies showing neurodevelopmental effects at similar levels without causing overt toxicity. While the reproductive toxicity NOAEL for BPA is lower (around 50 μ g/kg/day), our focus was on assessing broader developmental and neural effects of BPA exposure rather than solely reproductive outcomes. The use of the systemic NOAEL dose allowed us to investigate the effects on neural and systemic endpoints, particularly because prenatal BPA exposure has been shown to affect multiple organ systems, including the developing nervous system, at doses exceeding the reproductive NOAEL^{78,79}.

We acknowledge that lower, environmentally relevant doses of BPA might produce different or more subtle effects. Numerous studies have shown that even low-level BPA exposure can alter neurodevelopment⁸⁰, though possibly through mechanisms distinct from those observed at higher doses. Our choice of a high dose enabled us to identify robust alterations in NSC proliferation, gene expression, and neurosphere growth. Nonetheless, our findings underscore the need for future studies to explore a range of BPA exposure levels, particularly those closer to typical environmental exposure, to better understand the dose–response relationship and determine whether the effects seen at higher doses translate to more common, low-dose scenarios.

Additionally, it is possible that lower doses of BPA may elicit non-monotonic dose responses, as reported in several endocrine-disrupting chemical (EDC) studies⁸¹. Such responses can result in qualitatively different effects at low doses compared to high doses. This phenomenon further underscores the importance of studying multiple doses in future experiments to better capture the spectrum of BPA's effects on neurodevelopment. Including lower doses that mimic environmental exposure would also provide insights into how BPA may affect human health, especially during critical periods of brain development. In future research, we aim to address the limitation of dose selection by including additional dose groups that reflect common human exposure levels (e.g., 0.05 mg/kg/day, aligned with typical environmental exposure). This expanded dosing range will provide a more comprehensive perspective on how BPA may impact neural development across varied exposure scenarios.

The potential transposition of these results to humans is an important consideration. Although this study is based on rat models, many of the differentially expressed genes (DEGs) we identified, such as *Atp1a3*⁸², *Grin1*^{83,84}, and *Gabrb1*⁸⁵, have human orthologs involved in similar neurodevelopmental pathways, supporting the relevance of our findings to human ASD. The sex-specific differences observed in our rat model may reflect

hormonal and epigenetic mechanisms that could also apply to humans, suggesting prenatal BPA exposure may affect male and female neurodevelopment differently. Future studies using human-derived stem cells or organoid models, along with protein-level validation of the DEGs, will be crucial to confirm the translational relevance of these findings and further explore how prenatal BPA exposure influences human neurodevelopment and ASD risk.

Our finding of increased neurosphere size in females, despite the higher prevalence of ASD-related gene expression in males, may reflect sex-specific neurodevelopmental differences, as highlighted in recent research on cortical structure in autism. A 2024 longitudinal study found that autistic females exhibit a thicker cortex at an early age and a faster cortical thinning trajectory across childhood, suggesting that female neurobiology involves unique structural adaptations not seen in males⁸⁶. These findings may indicate that females with autism display compensatory or alternative growth responses, which could also manifest at the cellular level in NSCs, resulting in the larger neurospheres observed in our study.

In this study, we explored how prenatal BPA exposure affects hippocampal NSC function, revealing notable sex-specific differences in neurosphere characteristics. Our findings align with those of Thongkorn et al.³⁹, who demonstrated that prenatal BPA exposure influences cellular density, neuronal density, and neuronal composition in the hippocampus of offspring in a sex-dependent manner. Thongkorn et al.³⁹ observed that BPA exposure led to an increase in hippocampal size and a higher percentage of neuronal cells in several hippocampal regions in females, while males exhibited reduced neuronal density and a lower percentage of neuronal cells, particularly in the CA1, CA2/3, and DG regions⁴⁰. These changes are consistent with the upregulation of genes such as *Mief2* and *Eif3h*, which were observed specifically in males and may contribute to cellular stress responses that compromise neuronal viability. Combined with our findings on NSC behavior under BPA exposure, these results underscore the complex sex-dependent effects of BPA on hippocampal development, suggesting that female offspring may exhibit resilience through adaptive cellular responses, while male offspring may be more susceptible to neurodevelopmental disruptions. These cumulative insights, alongside our previous work showing that prenatal BPA exposure impacts hippocampal cell numbers in offspring, highlight the importance of considering sex as a biological variable in neurodevelopmental toxicology and underscore the need for further studies to elucidate the pathways behind these differential responses.

In summary, prenatal BPA exposure significantly alters the transcriptome of genes associated with ASD in hippocampal NSCs, affecting neurological functions and revealing sex-specific changes in NSC proliferation, with males showing a decrease and females an increase. These findings emphasize the importance of sex-dependent influences on neurogenesis and neuronal differentiation in hippocampal development. While this study utilized a high dose of BPA to maximize the detection of neurodevelopmental changes, future research should explore lower, environmentally relevant doses to determine if the effects observed extend to typical exposure levels, offering a more comprehensive understanding of BPA's impact on NSCs and ASD-related genes (Fig. 10).

Methods

Animal husbandry and treatment

Wistar rats were purchased from the National Laboratory Animal Center (NLAC), Mahidol University, Thailand. All animals were housed at the Chulalongkorn University Laboratory Animal Center (CULAC) under standard temperature (20–26 °C) and humidity (30–70%) conditions in a 12-h light/dark cycle. Female rats were divided into 2 groups: the control group and the BPA treatment group. The weight of each rat was measured daily and was used to calculate the amount of BPA or vehicle control needed to treat each rat. Animal treatment was carried out and processed following the previously published studies³⁹. Briefly, for BPA treatment, we prepared BPA stock solution at a concentration of 250 mg/ml by dissolving BPA (Sigma-Aldrich, USA) in molecular grade absolute ethanol (Merck Millipore, USA) and then further diluted the BPA stock solution with corn oil to a final concentration of 5,000 µg/kg-maternal BW to treat each rat. For the vehicle control treatment, we prepared the vehicle control by diluting only molecular grade absolute ethanol with corn oil in amounts equivalent to those used in the BPA preparation, which was 90% corn oil to 10% ethanol. After mating, either BPA or the vehicle control was administered to each female rat once daily from gestational day 1 until parturition by intragastric feeding method using oral gavage. Rats used in the experiments were individually raised in separate ventilated cages in specific pathogen-free (SPF) conditions. Also, the sets of stainless-steel oral gavage needles used for intragastric feeding were separated in order to prevent cross-contamination between conditions. All reusable materials were cleaned with 70% ethanol and rinsed with copious amounts of Milli-Q deionized water before use. All experimental procedures were approved by the Chulalongkorn University Animal Care and Use Committee (Animal Use Protocol No. 1673007 and No. 1773011) of Chulalongkorn University. We confirm that all experiments were performed in accordance with the relevant guidelines and regulations, and the study is reported in accordance with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

NSC isolation and culture

According to the American Veterinary Medical Association (AVMA), postnatal day 1 neonatal rat pups were anesthetized with 150 mg/kg BW sodium pentobarbital, followed by hypothermia. To ensure death, decapitation, one of the AVMA-approved physical methods, was performed after cleaning neonatal rat pups with 70% ethanol. A prechilled 60-mm petri dish containing a dissecting medium to which the brain was rapidly removed and transferred was placed under a dissecting microscope (Nikon SMZ18 Stereo Microscope). Dissecting/dissociation medium was prepared following previously published protocol^{87,88}. The meninges were completely removed, the brain was then dissected under a microscope (Nikon SMZ18 Stereo Microscope), and the hippocampal tissues were collected in a 15-ml tube filled with dissecting medium. Hippocampal NSC culture was carried out and processed following the previously published studies⁸⁸. Briefly, the tissues were centrifuged

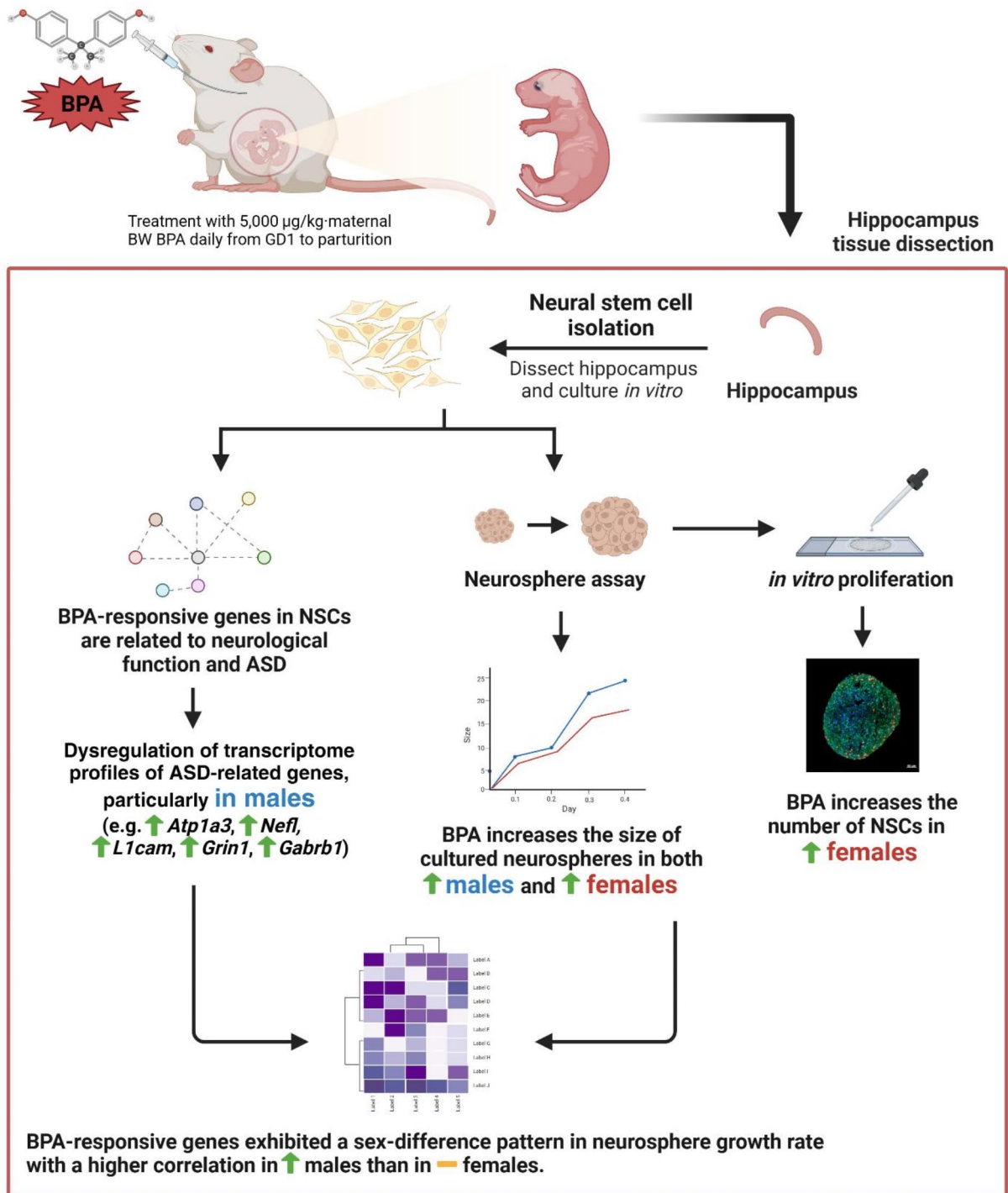


Fig. 10. Graphical abstract. Created with BioRender.com.

at 200 g for 1 min at room temperature, and then enzyme trypsin and DNase were added. Clumps of cells were broken up by performing trituration by gently and consistently pipetting up and down several times. The cells were washed with N2 medium to inactivate the enzyme. The isolated hippocampal NSCs were cultured in an initial proliferation medium (IPM) and formed neurospheres within 1–2 weeks^{88,89}. To characterize NSCs within neurospheres, 14-day cultured neurospheres were fixed with 4% paraformaldehyde and processed as described in the previous study⁹⁰. The embedded neurospheres were cryosectioned, washed with 1X Tris-buffered saline (TBST) 3 times, performing antigen retrieval 30 min at 90–95 °C, wash 3 times with 1X TBST, DNA hydrolysis

with 2N HCl 37 °C 10 min, neutralized with 0.1 M Sodium borate buffer pH8.5 10 min room temperature (RT), wash 3 times with 1X TBST, permeabilization using 0.25% Triton X-100 in 1X TBST, wash 3 times with 1X TBST and blocked with 3% Bovine Serum Albumin (BSA) in 1X TBST RT 1 h. After blocking, the spheres were incubated with a mouse monoclonal primary antibody against Sox2 to characterize neural progenitor cells. The spheres were washed with 1X TBST and incubated with the secondary antibody. The spheres were observed using a fluorescence microscope (Fig. 1). Apart from immunofluorescence staining of neurospheres, cells were preserved in RNAlater and stored at -80 °C until use.

RNA isolation and transcriptome profiling analysis

The hippocampal NSCs (BPA male n = 3, BPA female n = 3, Control male n = 3, Control female n = 3; neonatal rats in the same treatment came from independent replicates) were isolated and were cultured for 1–2 weeks to form neurospheres as described in the previous studies⁸⁸. Total RNA was purified from cultured neurospheres using the mirVana miRNA Isolation Kit (Ambion, USA) according to the manufacturer's protocol. The RNA yield and integrity were assessed using a Qubit 3.0 fluorometric quantification system and an Agilent 2100 Bioanalyzer, respectively. To identify DEGs in the hippocampal NSCs in response to prenatal BPA exposure, a transcriptome profiling analysis of total RNA isolated from the hippocampal NSCs of neonatal rat pups prenatally exposed to BPA or the vehicle control was performed by Vishuo Biomedical (Thailand) LTD., using the Illumina HiSeq platform in a 2 × 150 bp paired-end (PE) configuration; 6.0 Gb of raw data were generated per sample. The raw sequencing reads were in FASTQ formatted files. The samples were sequenced in PE mode with a length of 150 bases. The quality of the raw sequencing reads was assessed with FastQC v.0.11.5 software, and the surviving PE reads were aligned to the *Rattus norvegicus* reference genome (HISAT2 index: Ensembl Rnor_6.0 genome_tran). The alignment files were then analyzed individually with the Cufflinks v.2.1 software to detect and quantify previously known transcripts and to discover new transcripts or isoforms. Next, individual annotation files generated from the transcript assembly process were merged together to compare the expression levels among multiple samples or conditions. The expression fold change between each comparison group was calculated, and log transformed. Comparisons were performed with all male and female pups with the same treatment conditions combined into one group and then separated by sex.

Prediction of biological functions and interactome analysis

Ingenuity Pathway Analysis (IPA) software (version 22.0, Qiagen Inc., USA) is the powerful analysis and search tool that we used to predict biological functions, biological disorders, canonical pathways, and interactome networks, including key regulator identification and downstream effect prediction. The list of DEGs from RNA-seq overlapped with the list of genes validated to be associated with each function, disorder, and canonical pathway in the Ingenuity's Knowledge Base and created an interactome model/network. Statistical analysis was performed using Fisher's exact test to calculate the P-values, and a P-value < 0.05 was considered statistically significant.

Transcriptome data collection

The transcriptome profiling data of cells from individuals with ASD and cognitively normal control individuals were obtained from the NCBI GEO DataSets database (<http://www.ncbi.nlm.nih.gov/gds>) using the keywords "autism," "neural stem cells," "proliferation," and the following criteria: the included studies must be ASD studies; the studies must include cell-based gene expression profiles from microarray experiments; the experimental models were stem cells, primary cells, or cell lines; and each treatment group consisted of more than three samples. All "Supplementary Information", including series matrix files and related platforms, was freely available in the GEO DataSets database.

Hypergeometric distribution analysis

To determine whether the BPA-responsive genes in the hippocampal NSCs identified by RNA-seq were significantly associated with ASD candidate genes, we selected significant differentially expressed genes (DEGs) from RNA-seq results considering that DEGs should have a statistical value, both P-value and Q-value, less than 0.05. The list of differentially expressed BPA-responsive genes overlapped with the list of ASD candidate genes from ASD databases (SFARI and AutismKB). To further determine the association between these two lists of genes, we performed a hypergeometric distribution analysis using the hypergeometric distribution calculator program provided by Keisan Online Calculator (<https://keisan.casio.com/exec/system/1180573201>).

Quantitative RT-PCR analysis

DEGs in the hippocampal NSCs identified by RNA-seq transcriptome analysis were selected for further confirmation by quantitative RT-PCR analysis as previously described³⁹. Briefly, total RNA was isolated using the aforementioned method (BPA male n = 6, BPA female n = 6, control male n = 6, control male n = 6; neonatal rats in the same treatment came from independent replicates). RNA concentration was then measured using a Nanodrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA). Total RNA was used for cDNA synthesis with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Briefly, reaction buffer was first prepared by mixing 4 µl 5X Reaction Buffer, which was comprised of Tris-HCl (pH 8.3), KCl, MgCl₂, and DTT, with 1 µl (20 Unit) RiboLock RNase Inhibitor, 2 µl (20 nmol) dNTP Mix and 1 µl (200 Unit) RevertAid M-MuLV RT in a microcentrifuge tube, and perform cDNA synthesis as described in detail in our previous reports³⁹. Quantitative PCR analysis was conducted in triplicate using AccuPower® 2X GreenStar™ qPCR MasterMix (Bioneer, Korea) according to the manufacturer's instructions. Briefly, 1 µl of the cDNA was mixed with 2X Greenstar Master Mix, forward primer, reverse primer, and nuclease-free water. The reaction was then incubated in a Bio-Rad CFX Connect Real-Time System (Bio-

Rad, USA). The PCR amplification conditions and the expression level calculations were also described in detail in our previous reports³⁹. The specific primers in the qPCR analyses were designed using the UCSC Genome Browser (<https://genome.ucsc.edu/>), Ensembl Genome Browser [104] species *Rattus norvegicus* (Rnor_6.0) (https://may2021.archive.ensembl.org/Rattus_norvegicus/Info/Index), and Primer3 software version 0.4.0 (<http://bioinfo.ut.ee/primer3-0.4.0/>). The forward and reverse primers were designed for rat *Atp1a3*, *Nefl*, *L1cam*, *Grin1*, *Gabrb1*, and *Rn18s*. The sequences of the qPCR primers are shown in Supplementary Table S1.

Correlation analysis

Correlation analysis was performed to examine the possibility of the association between the gene expression level in response to BPA and the changes in the area of neurospheres. The expression value of selected DEGs obtained from qRT-PCR data and the mean area of neurospheres DIV0, DIV7, and DIV14 were used to plot the line. The slopes and the expression value were used to perform correlation analysis. R values of each correlation were determined and used to construct a heatmap in both sexes, males and females.

We also performed correlation analysis to examine the correspondence between the transcriptome profile of our in vitro NSCs and the transcriptome profile of in vivo NSC, basal progenitor, and newborn neurons from the database. Transcriptome dataset of in vivo NSC, basal progenitor, and newborn neuron was obtained from GSE134688. We thus combined the FPKM (Fragments Per Kilobase per Million mapped fragment) value of genes matched in both our RNA-seq data and GSE134688 dataset. The FPKM matrix was normalized using geometric mean normalization implemented in the DESeq package. The correlation of each in vivo transcriptome from individual cell types was correlated with our in vitro NSC transcriptome using Spearman's correlation. Moreover, the expression value of DEGs obtained from RNA-seq data and the mean area of the neurosphere were used to perform Pavlidis Template Matching (PTM) analysis⁹¹. The list of DEGs, R values, and P-values was determined.

In vitro proliferation assay

NSC isolation and culture were performed as described earlier in this study. Briefly, the isolated hippocampal NSCs were cultured in an initial proliferation medium (IPM), a Neurobasal medium minus phenol red (Invitrogen) supplemented with B27 serum-free supplement (Invitrogen), GlutaMAX I supplement (Invitrogen), Human FGF-Basic (PeproTech), Human EGF (PeproTech), and Antibiotic-antimycotic (Invitrogen), resulting in the formation of neurospheres within 1 to 2 weeks. A 10 mM stock solution of BrdU (ab142567) was prepared as described in the manufacturer's protocol. Briefly, a 10 mM BrdU stock solution was diluted 1000-fold with the cell culture medium (IPM) to make a 10 μ M BrdU labeling solution. During culture, on the day in vitro 14 (DIV14), the existing culture medium from the cells was removed and was replaced with the BrdU labeling solution, followed by incubating the cells with the BrdU labeling solution for 24 h in the CO₂ incubator at 37°C. Cultured neurospheres were fixed with 4% paraformaldehyde and processed as described in the previous study. The embedded neurospheres were cryosectioned using a cryostat (Leica CM1950). A 10- μ m thickness of neurosphere sections was washed with 1X TBST 3 times, followed by antigen retrieval, heat-induced epitope retrieval (HIER) using sodium citrate pH 6.0 as an antigen retrieval buffer, for 30 min at 90–95°C, wash 3 times with 1X TBST, DNA hydrolysis with 2N HCl 37°C 10 min, neutralized with 0.1M Sodium borate buffer pH8.5 10 min RT, wash 3 times with 1X TBST, and blocked with 3% BSA in 1X TBST RT 1 h. After blocking, the spheres were incubated with a monoclonal primary: anti-BrdU antibody (ab6326), which serves as a proliferation marker, and anti-Sox2 antibody (ab79351), a marker for neural stem/progenitor cells. Following incubation, the cryostat sections were washed three times for 5 min each with 1X TBST before incubated with the secondary antibodies labeled with Alexa Fluor-488 (ab150109) or Alexa Fluor-555 (ab150154) and counterstained with Hoechst33342.

Statistical analyses

Statistical analyses were conducted using SPSS version 22.0. The criterion for statistical significance was a P-value < 0.05. A t-test was used to determine the statistical significance of differences between the mean values of the two groups. Hypergeometric distribution analysis was performed to determine the association of DEGs with ASD candidate genes obtained from the SFARI (<https://gene.sfari.org/>) and AutismKB (<http://autismkb.cb.i.pku.edu.cn/>) databases and to determine the association of DEGs with other transcriptomic studies using the hypergeometric distribution calculator in the Keisan Online Calculator program (<http://keisan.casio.com/exec/system/1180573201>). A P-value < 0.05 was considered statistically significant.

Conclusion

This study significantly enhances our understanding of the impact of prenatal bisphenol A (BPA) exposure on NSC dynamics and its potential role in the pathogenesis of ASD. Our findings demonstrate that BPA acts as a critical environmental risk factor, leading to the dysregulation of ASD-related gene transcriptomes in NSCs. Notably, we identified sex-specific effects, with a marked correlation between BPA-induced gene expression changes and neurosphere size in male offspring, shedding light on the possible mechanisms behind the male bias in ASD prevalence. In both sexes, prenatal BPA exposure caused a significant reduction in NSC proliferation, further underscoring the disruption of neurogenesis.

This study highlights key molecular pathways linking BPA exposure to ASD, particularly through the identification of upregulated ASD-related genes, which may serve as potential therapeutic targets. Our integrative approach, combining transcriptome profiling, pathway analysis, and in vitro proliferation assays, provides a comprehensive view of the biological perturbations induced by BPA. The correlation between BPA-responsive DEGs and neurosphere characteristics, coupled with the significant changes observed in in vitro proliferation assays, underscores the potential influence of BPA on NSC dynamics and neurodevelopment. This comprehensive investigation provides valuable insights into the molecular underpinnings of BPA-induced effects on NSCs and

underscores the need for continued exploration into the complex interplay between environmental exposures and neurodevelopmental disorders, offering avenues for targeted interventions and preventative strategies.

Data availability

The transcriptome profiling data used in this study have been published in the NCBI GEO DataSets database (GSE65106, GSE67528, and GSE124308). The RNA-seq data in this study is also publicly available in the NCBI GEO DataSets database.

Received: 27 June 2024; Accepted: 10 January 2025

Published online: 22 January 2025

References

1. American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders (DSM-5*)* (American Psychiatric Pub, 2013).
2. Maenner, M. J. et al. Prevalence and characteristics of autism spectrum disorder among children aged 8 years—Autism and developmental disabilities monitoring network, 11 Sites, United States, 2020. *Morb. Mortal. Wkly. Rep. Surveill. Summ.* **72**, 1–14. <https://doi.org/10.15585/mmwr.ss7202a1> (2023).
3. Maenner, M. J. et al. Prevalence and characteristics of autism spectrum disorder among children aged 8 years—Autism and developmental disabilities monitoring network, 11 Sites, United States, 2018. *Morb. Mortal. Wkly. Rep. Surveill. Summ.* **70**, 1–16. <https://doi.org/10.15585/mmwr.ss7011a1> (2021).
4. Giarelli, E. et al. Sex differences in the evaluation and diagnosis of autism spectrum disorders among children. *Disabil. Health J.* **3**, 107–116. <https://doi.org/10.1016/j.dhjo.2009.07.001> (2010).
5. Loomes, R., Hull, L. & Mandy, W. P. L. What Is the male-to-female ratio in autism spectrum disorder? A systematic review and meta-analysis. *J. Am. Acad. Child Adolesc. Psychiatry* **56**, 466–474. <https://doi.org/10.1016/j.jaac.2017.03.013> (2017).
6. Saechua, C. et al. Impact of gene polymorphisms involved in the vitamin D metabolic pathway on the susceptibility to and severity of autism spectrum disorder. *Sci. Rep.* **14**, 28333. <https://doi.org/10.1038/s41598-024-79994-9> (2024).
7. Saeliw, T. et al. Investigation of chimeric transcripts derived from LINE-1 and Alu retrotransposons in cerebellar tissues of individuals with autism spectrum disorder (ASD). *Sci. Rep.* **14**, 21889 (2024). <https://doi.org/10.1038/s41598-024-72334-x>
8. Saeliw, T. et al. LINE-1 and Alu methylation signatures in autism spectrum disorder and their associations with the expression of autism-related genes. *Sci. Rep.* **12**, 13970 <https://doi.org/10.1038/s41598-022-18232-6> (2022).
9. Saeliw, T. et al. Integrated genome-wide Alu methylation and transcriptome profiling analyses reveal novel epigenetic regulatory networks associated with autism spectrum disorder. **9**, 27. <https://doi.org/10.1186/s13229-018-0213-9> (2018).
10. Tanguansri, C. et al. Investigation of epigenetic regulatory networks associated with autism spectrum disorder (ASD) by integrated global LINE-1 methylation and gene expression profiling analyses. *PLoS ONE* **13**, e0201071–e0201071. <https://doi.org/10.1371/journal.pone.0201071> (2018).
11. Sun, W. et al. Histone acetylome-wide association study of autism spectrum disorder. *Cell* **167**, 1385–1397.e1311. <https://doi.org/10.1016/j.cell.2016.10.031> (2016).
12. Smith, R. & Sadee, W. Synaptic signaling and aberrant RNA splicing in autism spectrum disorders. **3**. <https://doi.org/10.3389/fnsy.n.2011.00001> (2011).
13. Sarachana, T., Zhou, R., Chen, G., Manji, H. K. & Hu, V. W. Investigation of post-transcriptional gene regulatory networks associated with autism spectrum disorders by microRNA expression profiling of lymphoblastoid cell lines. *Genome Med.* **2**, 23. <https://doi.org/10.1186/gm144> (2010).
14. Moosa, A., Shu, H., Sarachana, T. & Hu, V. W. Are endocrine disrupting compounds environmental risk factors for autism spectrum disorder?. *Horm. Behav.* **101**, 13–21. <https://doi.org/10.1016/j.yhbeh.2017.10.003> (2018).
15. Saeliw, T. et al. Epigenetic gene-regulatory loci in Alu elements associated with autism susceptibility in the prefrontal cortex of ASD. *Int. J. Mol. Sci.* **24**. <https://doi.org/10.3390/ijms24087518> (2023).
16. Huguet, G., Benabou, M. & Bourgeron, T. In *A Time for Metabolism and Hormones* (eds Sassone-Corsi, P. & Christen, Y.) 101–129 (Springer, 2016).
17. Cunha, Y. G. O., do Amaral, G. C. B., Felix, A. A., Blumberg, B. & Amato, A. A. Early-life exposure to endocrine-disrupting chemicals and autistic traits in childhood and adolescence: A systematic review of epidemiological studies. *Front. Endocrinol.* **14**, 1184546. <https://doi.org/10.3389/fendo.2023.1184546> (2023).
18. Itoh, K., Yaoi, T. & Fushiki, S. Bisphenol A, an endocrine-disrupting chemical, and brain development. **32**, 447–457. <https://doi.org/10.1111/j.1440-1789.2011.01287.x> (2012).
19. Marinello, W. P. & Patisaul, H. B. Endocrine disrupting chemicals (EDCs) and placental function: Impact on fetal brain development. *Adv. Pharmacol.* **92**, 347–400. <https://doi.org/10.1016/bs.apha.2021.04.003> (2021).
20. Kanlayaprasit, S. et al. Sex-specific impacts of prenatal bisphenol A exposure on genes associated with cortical development, social behaviors, and autism in the offspring's prefrontal cortex. *Biol Sex Differ* **15**, 40 <https://doi.org/10.1186/s13293-024-00614-2> (2024).
21. Kim, K. et al. Potencies of bisphenol a on the neuronal differentiation and hippocampal neurogenesis. *J. Toxicol. Environ. Health Part A* **72**, 1343–1351. <https://doi.org/10.1080/15287390903212501> (2009).
22. Wolstenholme, J. T. et al. Gestational exposure to bisphenol a produces transgenerational changes in behaviors and gene expression. *Endocrinology* **153**, 3828–3838. <https://doi.org/10.1210/en.2012-1195> (2012).
23. Zhang, H. et al. Maternal exposure to environmental bisphenol A impairs the neurons in hippocampus across generations. *Toxicology* **432**, 152393. <https://doi.org/10.1016/j.tox.2020.152393> (2020).
24. Hwang, M., Park, S.-J. & Lee, H.-J. Risk assessment of bisphenol A in the Korean General Population. **13**, 3587 (2023)
25. Karmakar, P. C. et al. Paternal exposure to bisphenol-A transgenerationally impairs testis morphology, germ cell associations, and stemness properties of mouse spermatogonial stem cells. *Int. J. Mol. Sci.* **21**. <https://doi.org/10.3390/ijms21155408> (2020).
26. Tyl, R. W. et al. Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. *Toxicol. Sci.* **68**, 121–146. <https://doi.org/10.1093/toxsci/68.1.121> (2002).
27. Balakrishnan, B., Henare, K., Thorstensen, E. B., Ponnampalam, A. P. & Mitchell, M. D. Transfer of bisphenol A across the human placenta. *Am. J. Obstet. Gynecol.* **202**(393), e391–397. <https://doi.org/10.1016/j.ajog.2010.01.025> (2010).
28. Nishikawa, M. et al. Placental transfer of conjugated bisphenol A and subsequent reactivation in the Rat Fetus. *Environ. Health Perspect.* **118**, 1196–1203. <https://doi.org/10.1289/ehp.0901575> (2010).
29. Huang, R. P. et al. Bisphenol A concentrations in human urine, human intakes across six continents, and annual trends of average intakes in adult and child populations worldwide: A thorough literature review. *Sci. Total Environ.* **626**, 971–981. <https://doi.org/10.1016/j.scitotenv.2018.01.144> (2018).
30. Liu, J. et al. Bisphenol A metabolites and bisphenol S in paired maternal and cord serum. *Environ. Sci. Technol.* **51**, 2456–2463. <https://doi.org/10.1021/acs.est.6b05718> (2017).

31. Edlow, A. G., Chen, M., Smith, N. A., Lu, C. & McElrath, T. F. Fetal bisphenol A exposure: Concentration of conjugated and unconjugated bisphenol A in amniotic fluid in the second and third trimesters. *Reproduct. Toxicol.* **34**, 1–7. <https://doi.org/10.1016/j.reprotox.2012.03.009> (2012).
32. Schönfelder, G. et al. Parent bisphenol A accumulation in the human maternal-fetal-placental unit. *Environ. Health Perspect.* **110**, A703–707. <https://doi.org/10.1289/ehp.110-1241091> (2002).
33. Todaka, E. & Mori, C. Necessity to establish new risk assessment and risk communication for human fetal exposure to multiple endocrine disruptors in Japan. *Congenit. Anom.* **42**, 87–93. <https://doi.org/10.1111/j.1741-4520.2002.tb00857.x> (2002).
34. Kuruto-Niwa, R., Tateoka, Y., Usuki, Y. & Nozawa, R. Measurement of bisphenol A concentrations in human colostrum. *Chemosphere* **66**, 1160–1164. <https://doi.org/10.1016/j.chemosphere.2006.06.073> (2007).
35. Mendonca, K., Hauser, R., Calafat, A. M., Arbuckle, T. E. & Duty, S. M. Bisphenol A concentrations in maternal breast milk and infant urine. *Int. Arch. Occup. Environ. Health* **87**, 13–20. <https://doi.org/10.1007/s00420-012-0834-9> (2014).
36. Moriyama, K. et al. Thyroid hormone action is disrupted by bisphenol A as an antagonist. *J. Clin. Endocrinol. Metab.* **87**, 5185–5190. <https://doi.org/10.1210/jc.2002-020209> (2002).
37. Miyakoda, H., Tabata, M., Onodera, S. & Tkeda, K. Passage of bisphenol A into the fetus of the pregnant rat. *J. Health Sci.* **45**, 318–323. <https://doi.org/10.1248/jhs.45.318> (1999).
38. Kanlayaprasit, S. et al. Autism-related transcription factors underlying the sex-specific effects of prenatal bisphenol A exposure on transcriptome-interactome profiles in the offspring prefrontal cortex. *Int. J. Mol. Sci.* **22**. <https://doi.org/10.3390/ijms222413201> (2021).
39. Thongkorn, S. et al. Sex differences in the effects of prenatal bisphenol A exposure on genes associated with autism spectrum disorder in the hippocampus. *Sci. Rep.* **9**, 3038. <https://doi.org/10.1038/s41598-019-39386-w> (2019).
40. Thongkorn, S. et al. Sex differences in the effects of prenatal bisphenol A exposure on autism-related genes and their relationships with the hippocampus functions. *Sci. Rep.* **11**, 1241. <https://doi.org/10.1038/s41598-020-80390-2> (2021).
41. Welch, C. et al. Bisphenol A affects neurodevelopmental gene expression, cognitive function, and neuromuscular synaptic morphology in *Drosophila melanogaster*. *Neurotoxicology* **89**, 67–78. <https://doi.org/10.1016/j.neuro.2022.01.006> (2022).
42. Thongkorn, S. et al. Investigation of autism-related transcription factors underlying sex differences in the effects of bisphenol A on transcriptome profiles and synaptogenesis in the offspring hippocampus. *Biol. Sex Differ.* **14**, 8. <https://doi.org/10.1186/s13293-023-00496-w> (2023).
43. Singh, S. J. et al. Bisphenol-A (BPA) impairs hippocampal neurogenesis via inhibiting regulation of the ubiquitin proteasomal system. *Mol. Neurobiol.* **60**, 3277–3298. <https://doi.org/10.1007/s12035-023-03249-3> (2023).
44. Tiwari, S. K., Agarwal, S., Chauhan, L. K., Mishra, V. N. & Chaturvedi, R. K. Bisphenol-A impairs myelination potential during development in the hippocampus of the rat brain. *Mol. Neurobiol.* **51**, 1395–1416. <https://doi.org/10.1007/s12035-014-8817-3> (2015).
45. Wegiel, J. et al. The neuropathology of autism: defects of neurogenesis and neuronal migration, and dysplastic changes. **119**, 755–770. <https://doi.org/10.1007/s00401-010-0655-4> (2010).
46. Komada, M., Nagao, T. & Kagawa, N. Prenatal and postnatal bisphenol A exposure inhibits postnatal neurogenesis in the hippocampal dentate gyrus. *J. Toxicol. Sci.* **45**, 639–650. <https://doi.org/10.2131/jts.45.639> (2020).
47. Chung, L., Bey, A. L. & Jiang, Y. H. Synaptic plasticity in mouse models of autism spectrum disorders. *Korean J. Physiol. Pharmacol. Off. J. Korean Physiol. Soc. Korean Soc. Pharmacol.* **16**, 369–378. <https://doi.org/10.4196/kjpp.2012.16.6.369> (2012).
48. Horder, J. et al. GABA(A) receptor availability is not altered in adults with autism spectrum disorder or in mouse models. *Sci. Transl. Med.* **10**. <https://doi.org/10.1126/scitranslmed.aam8434> (2018).
49. Rojas, D. C. The role of glutamate and its receptors in autism and the use of glutamate receptor antagonists in treatment. *J. Neural Transm.* **121**, 891–905. <https://doi.org/10.1007/s00702-014-1216-0> (2014).
50. Ivanov, D. P., Parker, T. L., Walker, D. A., Alexander, C., Ashford, M. B., Gellert, P. R., Garnett, M. C., Multiplexing spheroid volume resazurin and acid phosphatase viability assays for high-throughput screening of tumour spheroids and stem cell neurospheres. *PLoS ONE* **9**(8), e103817. <https://doi.org/10.1371/journal.pone.0103817> (2014).
51. Halladay, A. K. et al. Sex and gender differences in autism spectrum disorder: summarizing evidence gaps and identifying emerging areas of priority. **6**, 36 <https://doi.org/10.1186/s13229-015-0019-y> (2015).
52. Weirling, D. M. & Geschwind, D. H. Sex differences in autism spectrum disorders. *Curr Opin Neurol* **26**, 146–153 <https://doi.org/10.1097/WCO.0b013e32835ee548> (2013).
53. Zhu, X. J. et al. Neuronal nitric oxide synthase-derived nitric oxide inhibits neurogenesis in the adult dentate gyrus by down-regulating cyclic AMP response element binding protein phosphorylation. *Neuroscience* **141**, 827–836. <https://doi.org/10.1016/j.neuroscience.2006.04.032> (2006).
54. Packer, M. A. et al. Nitric oxide negatively regulates mammalian adult neurogenesis. *Proceedings of the National Academy of Sciences* **100**, 9566. <https://doi.org/10.1073/pnas.1633579100> (2003).
55. Bowman, R. E. et al. Bisphenol-A exposure during adolescence leads to enduring alterations in cognition and dendritic spine density in adult male and female rats. *Hormones and behavior* **69**, 89–97 (2015). <https://doi.org/10.1016/j.yhbeh.2014.12.007> (2015).
56. Ji, L., Chauhan, A., Brown, W. T. & Chauhan, V. Increased activities of Na⁺/K⁺-ATPase and Ca²⁺/Mg²⁺-ATPase in the frontal cortex and cerebellum of autistic individuals. *Life Sci.* **85**, 788–793. <https://doi.org/10.1016/j.lfs.2009.10.008> (2009).
57. Hashem, S. et al. Genetics of structural and functional brain changes in autism spectrum disorder. *Translat. Psychiatry* **10**, 229. <https://doi.org/10.1038/s41398-020-00921-3> (2020).
58. Ziats, M. N. & Rennett, O. M. Expression Profiling of Autism Candidate Genes during Human Brain Development Implicates Central Immune Signaling Pathways. *PLOS ONE* **6**, e24691 (2011). <https://doi.org/10.1371/journal.pone.0024691>
59. Anitha, A. et al. Brain region-specific altered expression and association of mitochondria-related genes in autism. *Molecular autism* **3**, 12. <https://doi.org/10.1186/2040-2392-3-12> (2012).
60. Lee, W.-C., Chen, Y.-Y., Kan, D. & Chien, C.-L. A neuronal death model: overexpression of neuronal intermediate filament protein peripherin in PC12 cells. *J. Biomed. Sci.* **19**, 8. <https://doi.org/10.1186/1423-0127-19-8> (2012).
61. Kaplan, M. P., Chin, S. S., Flegner, K. H. & Liem, R. K. Alpha-internexin, a novel neuronal intermediate filament protein, precedes the low molecular weight neurofilament protein (NF-L) in the developing rat brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **10**, 2735–2748 <https://doi.org/10.1523/jneurosci.10-08-02735.1990> (1990).
62. Mohammad, M. H., Al-Shammari, A. M., Al-Juboory, A. A. & Yaseen, N. Y. Characterization of neural stemness status through the neurogenesis process for bone marrow mesenchymal stem cells. *Stem Cells Cloning* **9**, 1–15. <https://doi.org/10.2147/SCCAA.S94545> (2016).
63. Amar, M. et al. Autism-linked Cullin3 germline haploinsufficiency impacts cytoskeletal dynamics and cortical neurogenesis through RhoA signaling. *Molecular Psychiatry*. <https://doi.org/10.1038/s41380-021-01052-x> (2021).
64. Demyanenko, G. P., Tsai, A. Y. & Maness, P. F. Abnormalities in neuronal process extension, hippocampal development, and the ventricular system of L1 knockout mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **19**, 4907–4920. <https://doi.org/10.1523/jneurosci.19-12-04907.1999> (1999).
65. Son, Y. S. et al. Brief report: L1 cell adhesion molecule, a novel surface molecule of human embryonic stem cells, is essential for self-renewal and pluripotency. *Stem cells (Dayton, Ohio)* **29**, 2094–2099 (2011). <https://doi.org/10.1002/stem.754> (2011).
66. LoTurco, J. J., Blanton, M. G. & Kriegstein, A. R. Initial expression and endogenous activation of NMDA channels in early neocortical development. *J. Neurosci.* **11**, 792. <https://doi.org/10.1523/JNEUROSCI.11-03-00792.1991> (1991).

67. Bannerman, D. M. et al. Hippocampal synaptic plasticity, spatial memory and anxiety. *Nature Reviews Neuroscience* **15**, 181–192. <https://doi.org/10.1038/nrn3677> (2014).
68. Tashiro, A., Sandler, V. M., Toni, N., Zhao, C. & Gage, F. H. NMDA-receptor-mediated, cell-specific integration of new neurons in adult dentate gyrus. *Nature* **442**, 929–933. <https://doi.org/10.1038/nature05028> (2006).
69. Luk, K. C., Kennedy, T. E. & Sadikot, A. F. Glutamate Promotes Proliferation of Striatal Neuronal Progenitors by an NMDA Receptor-Mediated Mechanism. *The Journal of Neuroscience* **23**, 2239. <https://doi.org/10.1523/JNEUROSCI.23-06-02239.2003> (2003)
70. Fatemi, S. H. et al. mRNA and protein levels for GABAA α 4, α 5, β 1 and GABABR1 receptors are altered in brains from subjects with autism. *J. Autism. Dev. Disord.* **40**, 743–750. <https://doi.org/10.1007/s10803-009-0924-z> (2010).
71. Haydar, T. F., Wang, F., Schwartz, M. L. & Rakic, P. Differential modulation of proliferation in the neocortical ventricular and subventricular zones. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **20**, 5764–5774. <https://doi.org/10.1523/jneurosci.20-15-05764.2000> (2000).
72. Yadav, R., Deepshikha, D. & Srivastava, P. Homology Modeling and Protein Interaction Map of CHRNA7 Neurogenesis Protein. *Annals of Neurosciences* **24**, 173–179. <https://doi.org/10.1159/000477155> (2017).
73. Liang, R. et al. Aquaporin-4 Mediates the Suppressive Effect of Lipopolysaccharide on Hippocampal Neurogenesis. *Neuroimmunomodulation* **23**, 309–317. <https://doi.org/10.1159/000467141> (2016)
74. Hoyt, K. R., Horning, P., Georgette Ang, P., Karelina, K. & Obrietan, K. Ribosomal S6 kinase signaling regulates neuronal viability during development and confers resistance to excitotoxic cell death in mature neurons. *Neuroscience* **558**, 1–10. <https://doi.org/10.1016/j.neuroscience.2024.08.012> (2024).
75. Okada, M. et al. Estrogen Stimulates Proliferation and Differentiation of Neural Stem/Progenitor Cells through Different Signal Transduction Pathways. *International journal of molecular sciences* **11**, 4114–4123 (2010).
76. Ábrahám, I. M. & Herbison, A. E. Major sex differences in non-genomic estrogen actions on intracellular signaling in mouse brain in vivo. *Neuroscience* **131**, 945–951. <https://doi.org/10.1016/j.neuroscience.2004.10.046> (2005).
77. Nayan, N. M., Husin, A. & Siran, R. The risk of prenatal bisphenol A exposure in early life neurodevelopment: Insights from epigenetic regulation. *Early Human Development* **198**, 106120 <https://doi.org/10.1016/j.earlhumdev.2024.106120> (2024).
78. Prata, J. C., da Costa, J. P., Lopes, I., Duarte, A. C. & Rocha-Santos, T. Environmental exposure to microplastics: An overview on possible human health effects. *Science of The Total Environment* **702**, 134455 (2020). <https://doi.org/https://doi.org/10.1016/j.scitotenv.2019.134455>
79. Ragusa, A. et al. Placententa: First evidence of microplastics in human placenta. *Environ Int* **146**, 106274 <https://doi.org/10.1016/j.envint.2020.106274> (2021).
80. Tiwari, S. K., Agarwal, S., Seth, A., Yadav, B., Ray, R. S., Mishra, V. N., Chaturvedi, R. K. Inhibitory effects of bisphenol-A on neural stem cells proliferation and differentiation in the rat brain are dependent on Wnt/ β -Catenin pathway. *Mole. Neurobiol.* **52**(3), 1735–1757. <https://doi.org/10.1007/s12035-014-8940-1> (2015).
81. Rubin, B. S. Bisphenol A: An endocrine disruptor with widespread exposure and multiple effects. *J. Steroid Biochem. Mol. Biol.* **127**, 27–34. <https://doi.org/10.1016/j.jsbmb.2011.05.002> (2011).
82. Torres, A. et al. De novo ATP1A3 and compound heterozygous NLRP3 mutations in a child with autism spectrum disorder, episodic fatigue and somnolence, and muckle-wells syndrome. *Mol. Genet. Metab. Rep.* **16**, 23–29. <https://doi.org/10.1016/j.ymgmr.2018.06.001> (2018).
83. Rossi, M. et al. Novel homozygous missense variant of GRIN1 in two sibs with intellectual disability and autistic features without epilepsy. *Eur. J. Hum. Genet.* **25**, 376–380. <https://doi.org/10.1038/ejhg.2016.163> (2017).
84. Lemke, J. R. et al. Delineating the GRIN1 phenotypic spectrum: A distinct genetic NMDA receptor encephalopathy. *Neurology* **86**, 2171–2178. <https://doi.org/10.1212/wnl.0000000000002740> (2016).
85. Hussman, J. P. Suppressed GABAergic inhibition as a common factor in suspected etiologies of autism. *J. Autism Dev. Disord.* **31**, 247–248. <https://doi.org/10.1023/a:1010715619091> (2001).
86. Andrews, D. S. et al. Sex differences in trajectories of cortical development in autistic children from 2–13 years of age. *Mol. Psychiatry* **29**, 3440–3451. <https://doi.org/10.1038/s41380-024-02592-8> (2024).
87. Beaudoin, G. M. 3rd. et al. Culturing pyramidal neurons from the early postnatal mouse hippocampus and cortex. *Nat. Protocols* **7**, 1741–1754. <https://doi.org/10.1038/nprot.2012.099> (2012).
88. Guo, W., Patzlaff, N. E., Jobe, E. M. & Zhao, X. Isolation of multipotent neural stem or progenitor cells from both the dentate gyrus and subventricular zone of a single adult mouse. *Nat. Protocols* **7**, 2005–2012. <https://doi.org/10.1038/nprot.2012.123> (2012).
89. Xu, W. et al. Genome-wide transcriptome landscape of embryonic brain-derived neural stem cells exposed to alcohol with strain-specific cross-examination in BL6 and CD1 mice. *Sci. Rep.* **9**, 206. <https://doi.org/10.1038/s41598-018-36059-y> (2019).
90. Guerrero-Cázares, H., Chaichana, K. L. & Quiñones-Hinojosa, A. Neurosphere culture and human organotypic model to evaluate brain tumor stem cells. *Methods Mol. Biol.* **568**, 73–83. https://doi.org/10.1007/978-1-59745-280-9_6 (2009).
91. Pavlidis, P. & Noble, W. S. Analysis of strain and regional variation in gene expression in mouse brain. *Genome Biol.* **2**, Research0042. <https://doi.org/10.1186/gb-2001-2-10-research0042> (2001).

Acknowledgements

KK, PL, and PP are graduate students in the Ph.D. Program in Clinical Biochemistry and Molecular Medicine, Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University. ST is a post-doctoral researcher in the Department of Biotechnology and Biomedicine (DTU Bioengineering), Technical University of Denmark. SK and TSa are The Second Century Fund Office (C2F) postdoctoral researchers in the Chulalongkorn Autism Research and Innovation Center of Excellence (Chula ACE), Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University. We wish to acknowledge Asst. Prof. Dr. Suwanakiet Sawangkoon, Assoc. Prof. Dr. Anusak Kijtaornrat, Dr. Nitira Anakul, Dr. Choopet Nitsakulthong, Ms. Sornsawan Chumjai, Ms. Saifon Sreechomphoe, Mr. Phanupong Dungkhokkrut of the Chulalongkorn University Laboratory Animal Center for their help with the ethics approval process and training KK, ST, SK, and TS in the proper care and use of laboratory animals. We wish to acknowledge Dr. Bumpenporn Sanannam, Ms. Kwanjira Songsritaya, Ms. Wasana Yuwattana, Mr. Thanawin Jantheang for helping in the animal experiment.

Author contributions

KK performed all the experiments, analyzed the data, prepared figures, and drafted the manuscript under the supervision of TS, NO, TK, and DJ. ST, SK, PL, and PP assisted KK in animal experiments, isolated tissues, and RNA. TSa performed the correlation analysis. ST performed the neurosphere assay. TS conceived of the study, designed the experiments, analyzed the data, interpreted the results, determined the conclusion. TS was responsible for funding acquisition and project administration. Resources were provided by VWH and TS, and

biological pathway analysis was led by VWH. KK and TS contributed to the writing of the original draft. Writing–review and editing was carried out by ST, SK, TSa, DJ, VWH, TK, NO, and TS. All the authors read and approved the final manuscript.

Funding

This research was supported by Ratchadapisek Somphot Fund for Supporting Center of Excellence, Chulalongkorn University, the Faculty of Allied Health Sciences Research Fund, Chulalongkorn University (AHS-CU 61004) to TS. This research was also supported by Thailand Science Research and Innovation Fund Chulalongkorn University (HEAF67370092 and HEA_FF_68_083_3700_006), Ratchadapisek Somphot Fund for Supporting Research Unit and Center of Excellence, Chulalongkorn University (CE66_046_3700_003), and the NSRF via the Program Management Unit for Human Resources & Institutional Development, Research and Innovation (B36G660008) to TS. The conclusions in this article were made by the authors. The Thailand Research Fund and Office of the Higher Education Commission may not necessarily agree. This research was also supported by Chulalongkorn University Laboratory Animal Center (CULAC) Grant (Animal use protocol No.1773011 and 2073011) to TS, SK and KK. KK was financially supported by "The 90th Anniversary Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund: GCUGR1125632109D-109)", "The Overseas Research Experience Scholarship for Graduate Students from the Graduate School, Chulalongkorn University" and "The 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship", Graduate School, Chulalongkorn University. This research and innovation activity were also funded by the National Research Council of Thailand (NRCT) to ST, PP, KK, PL, and TSa. ST received the Royal Golden Jubilee Ph.D. Programme Scholarship (Grant No. PHD/0029/2561) by the Thailand Research Fund and National Research Council of Thailand and was financially supported by "The 90th Anniversary Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund: GCUGR1125632108D-108)", Graduate School, Chulalongkorn University. SK and TSa was financially supported by "The Second Century Fund (C2F)", Chulalongkorn University, Bangkok, Thailand. PP and PL were supported by "The 90th Anniversary Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund: GCUGR1125651060D-060, and GCUGR1125651062D-062)" respectively from the Graduate School, Chulalongkorn University.

Declarations

Ethics approval and consent to participate

All animal experimental procedures were approved by the Chulalongkorn University Animal Care and Use Committee (Animal Use Protocol No. 1673007 and No. 1773011), Chulalongkorn University.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-86392-2>.

Correspondence and requests for materials should be addressed to T.S.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

© The Author(s) 2025