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Human-induced pluripotent stem cell-derived in vitro neuronal models for the investigation of environmental alterations on embryonic brain development

The Thesis of the PhD dissertation

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1 INTRODUCTION & AIMS

The Developmental Origins of Health and Disease (DOHaD) concept associates altered environmental conditions during the periconceptional period (PC), such as chemical stressors during pregnancy and assisted reproductive technologies (ART), to an individual's health and metabolic condition throughout their lifetime. Currently there is a global increase in the prevalence of non-communicable diseases (NCDs), including neurological disorders, affecting millions of people annually. Thus, there is an urgent need for human relevant models that can provide translatable information to assist in NCD treatment and/or prevention. Environmental disturbances to the brain during vulnerable developmental stages can lead to aberrations to brain structure, connectivity, and function, with substantial consequences. The susceptibility of the foetal brain to environmental insults is evident, and exposure to environmental chemicals can result in the disruption of the earliest processes in embryonic brain development form neurulation through to synaptogenesis. Increasing evidence suggests that the potential adverse neurological outcomes that can arise due to environmental challenges during the foetal stages of neurodevelopment include the onset of neurodevelopmental disorders (NDDs) such as autism spectrum disorders (ASD) and intellectual disability, as well as neurodegenerative diseases (NDs), such as Alzheimer's disease (AD).

Epigenetic mechanisms have been proposed to provide a further mechanistic explanation between DOHaD-related environmental exposures and phenotypic alterations. Most evidence in support of the DOHaD concept was obtained from animal models and human observational studies. To date, human based DOHaD investigations have relied upon children conceived via ART technologies; an ever-growing clinical cohort that includes millions of people globally. The in vitro procedures required to carry out ART treatments, such as embryo culture and in vitro fertilization (IVF) expose gametes and preimplantation embryos to unsuitable environmental conditions that may influence the physiological and metabolic phenotype of the offspring. Epigenetic alterations underlie ART-induced aberrations to the long-term health of progeny, and children born after ART procedures are at an elevated risk of imprinting defects caused by DNA methylation errors. Moreover, a growing number of studies suggest an association between ART and imprinting disorders (ID), including the NDDs Angelman syndrome (AS) and Prader-Willi syndrome (PWS), as well as Silver-Russell syndrome (SRS), which also induces aberrations to neurodevelopment. The ever-increasing rate of ART-facilitated pregnancies is accompanied by an urgent need for improved models to further understand ART-induced epigenetic defects and its association to AS, PWS and SRS, which all have detrimental effects on the developing brain.

Bisphenol A (BPA) is an endocrine disrupting compound (EDC) that has garnered much attention in the scientific community due to its abundance in the environment and its potential hazardous effect on humans. BPA is a plastic component with a wide range of applications in modern life and is commonly utilised in several industries for the manufacturing of food and drink containers, epoxy resins, and polycarbonate plastics. BPA contamination has been detected throughout the environment in water, dust, soil and air, and human exposure can be facilitated via dietary ingestion from contaminated food products, dermal absorption, or inhalation. Current reports suggest that human exposure to BPA is linked to behavioural alterations and impaired cognitive function, and additionally, to NDDs such as schizophrenia and ASD, and NDs including AD.

Currently, there is a lack of human relevant data available to clarify BPA's effects on the developing embryonic brain, and previous studies have showed conflicting results. Therefore, human in vitro models that can offer further insight into the molecular effects of BPA on the developing human brain are required, to enhance the understanding of the consequences of BPA exposure on brain development and its relation to NDDs and NDs.

Human-induced pluripotent stem cells (hiPSCs), derived via the reprogramming of somatic cells, are defined by their capacity for self-renewal, proliferation, and differentiation into each of the embryonic germ layers. hiPSC-derived in vitro neural models can provide vital knowledge of the underlying mechanisms of neurological pathologies and could also be useful for the development of patient-specific therapies. HiPSC-derived NSCs are self-renewable, and they can be differentiated into several neuronal/glial subtypes of the brain, therefore, they can be used to replicate distinct developmental stages of human brain development. For instance, the neural induction of hiPSCs to NSCs in vitro is representative of the neurulation stage of embryonic brain development, from which NSCs first emerge during the development of the brain.

In the present two-part study, we firstly reviewed and critically assessed the suitability of hiPSCs for modelling ART-associated imprinting disorders that affect brain development. In addition, we reviewed the methylation and imprinting status of hiPSCs in culture and animal models of ART to determine patterns of vulnerability in imprinted regions of the genome. An improved mechanistic understanding of the loss of regulation of imprinted genes in hiPSCs enabled us to elucidate the epigenetic mechanisms underlying IDs and how they can be altered by ART procedures. Secondly, we established a novel in vitro 3D model for early CNS development using the neural induction of hiPSCs to NSCs. The expression of critical neuroectodermal and neural lineage markers showed that our in vitro system can be used to assess neurodevelopmental toxicity of DOHaD-related environmental chemical exposures during the neurulation stages of brain development in a human cell-based model. Additionally, for the first time, we performed a repeated-dose exposure of abundant environmental chemical BPA during the in vitro 3D neural induction of hiPSCs to NSCs over a 21-day period. This enabled the clarification of BPA-induced perturbations to NSC characteristics, namely inhibited proliferation, after a longer-term BPA exposure than in previous in vitro studies. Additionally, we investigated proteome remodelling in BPA-treated NSCs using quantitative proteomics combined with a disease network analysis, revealing novel BPA-induced molecular alterations in NSCs that could be linked to modified NSC properties during brain development, and the pathophysiology of NDDs and NDs.

Objectives of this study

The overall aim of this study was to find answers to the following scientific questions:

- Are hiPSC-derived in vitro systems suitable for modelling ART-associated IDs?
- Are there patterns of methylation defects in cultured hiPSCs and animal models of ART?
- Can these patterns of vulnerability in imprinted regions of the genome clarify the links between ART procedures and IDs, as well as the underlying epigenetic mechanisms?
- What impact does a repeated-dose exposure of environmentally relevant BPA concentrations have on the 3D neural induction of hiPSCs to NSCs?
- Does BPA treatment affect the rate of the neural induction, or critical NSC characteristics, such as proliferation and clonogenicity?
- How does BPA treatment affect NSC proteome remodelling? Can proteome-wide changes elucidate the underlying mechanisms linking BPA exposure to NDs and NDDs?

Specific objectives of the research:

- Critical evaluation of the methylated and imprinted status of cultured hiPSCs and animal models of ART in current literature
- Identify patterns of susceptibility in imprinted genes to enhance the understanding of the association between ART and IDs
- Establishment and characterization of the in vitro 3D neural induction of hiPSCs to NSCs via the detection of neuroectodermal and neural lineage markers.
- Investigation of the effects of a repeated-dose, sub-cytotoxic BPA exposure on the rate of the neural induction of hiPSCs to NSCs and critical NSC characteristics
- Evaluate proteome-wide changes in BPA-treated NSCs.
- Analysis of protein-protein interaction networks to enhance the understanding of the molecular roles and interactions of BPA dysregulated proteins in disease.

2 MATERIALS AND METHODS

2.1 hiPSC culture

This work employed the SBAD2 hiPSC line, which was reprogrammed via non-integrative Sendai virus transduction from healthy adult dermal fibroblast (NHDF-Ad) cells (Lonza, Cat#: CC-2511, 51-year-old Caucasian male). In vitro cell maintenance was undertaken at 37°C in a humidified environment containing 5% CO2. Cells were maintained using mTeSR™1 media (Stem Cell Technologies) and BD Matrigel™ matrix (BD Biosciences) was used for plate coating. According to the manufacturer's procedure, cells were passaged every 5-7 days using EDTA (0.02% Versene, Cat#: BE17-711E, Lonza). A regular mycoplasma screening procedure was carried out using the Venor®GeM-Advance (Minerva Biolabs) Mycoplasma Detection Kit. Earlier characterization of the SBAD2 hiPSCs showed representative examples of normal stem cell traits, such as the expression of key pluripotency markers, colony shape, and karyotype (Fehér et al., 2022; Snijders et al., 2021).

2.2 3D Neural Induction

Dual SMAD inhibition was used to stimulate neuroectodermal development during the in vitro differentiation of hiPSCs to NSCs (Chambers et al., 2009; Shi et al., 2012). Upon reaching 90% confluence, the neural induction was initiated by replacing mTeSR™1 medium with neural induction medium (NIM; Neurobasal medium: DMEM/F12, supplemented with 1x N2, 2x B27, 100µM β-mercaptoethanol, 2mM glutamine, 1x non-essential amino acid (NEAA), 5µg/mL insulin) supplemented with 200nM LDN-193189 HCL (Selleck Chemicals, cat# S7507), 10µM SB431542, and 5ng/mL basic fibroblast growth factor (bFGF). On Prime Surface 96well V plates in 200L NIM the following day, hiPSCs were dissociated with Accutase® solution and seeded as single cells (10,000 cells/well) for spheroid formation. Every third day, NIM was replaced with a 75% media change in order to minimise the physical disturbance of the spheroids.

2.3 Neural differentiation of NPCs

Neuronal progenitor cells (NPCs) were plated on culture dishes coated with poly-L-ornithine/laminin (Sigma-Aldrich) and were cultured for proliferation and propagation in neuronal maintenance medium (NMM) (1:1 vol/vol DMEM:F12 and neurobasal medium, 1x N-2 supplement, 1x B-27 supplement, 1x non-essential amino acids, 1 mM L-glutamine, 50 U/ml penicillin/streptomycin) supplemented with 10 ng/ml of EGF and 10 ng/ml bFGF. To induce differentiation into mature neurons, NPCs were plated at a density of 40.000 cells/cm^2 for immunocytochemistry and 100.000 cells/cm^2 for Western blot and RT-qPCR experiments and were cultured in NMM without the addition of bFGF and EGF. The medium was changed every 3-4 days during the terminal differentiation that took place for 42 days.

2.4 BPA Treated 3D Neural Induction

BPA was dissolved in DMSO to a final concentration of 100mM to create the stock solution. Using NIM, BPA was further diluted to the proper experimental concentrations. Following the start of the 3D neural induction, repeated doses of 0.01µM, 0.1µM, and 1µM BPA were given to spheroids at each medium change for 3 weeks. To reduce spheroid disturbance, BPA treatments and media changes were carried out on every third day. 75% of the used medium was replaced with NIM that contained BPA concentrations that produced the desired treatment concentrations. NIM supplemented with 0.1% DMSO served as the experiment's vehicle control.

2.5 Immunocytochemical staining.

Fixation of 3D NSC spheroids was performed with 4% PFA in 0.1mol/L phosphate buffer for 1h at RT, followed by 3 washes with phosphate buffered saline (PBS). Spheroid permeabilization was carried with 0.2% TritonX-100 in PBS, followed by blocking in 3% BSA in PBS at RT for 1h. Primary antibody incubation was performed overnight at 4°C. Spheroids were then washed three times in PBS before being incubated with isotype-specific secondary antibodies diluted in 3% BSA in PBS for 1h at RT. Spheroids were then washed three times in PBS before being mounted using ProLongTM Diamond Antifade Mountant and DAPI for nuclear labeling on SuperfrostTM Ultra Plus Adhesion Slides from Thermo Fisher Scientific. A BX-41 epifluorescent microscope (objectives: 20x 0.50 NA; 40x 0.75 NA; Olympus) equipped with a DP-74 digital camera and Cellsens software (V1.18; Olympus) was used to acquire images.

2.6 Flow Cytometry

Cells were dissociated using Accutase® solution, washed 3x with PBS and subsequently transferred to flow cytometry tubes (Beckman Coulter, #2523749). Cells were then stained with Fixable Viability Dye eFluor™ 660 (eBioscience™ #65-0864) for 30 minutes at 2-8°C in the dark. Samples were then washed with 1% BSA solution, followed by centrifugation at 1500rpm at RT for 10 minutes. Staining was then carried out using the True-Nuclear™ transcription factor buffer set (BioLegend #424401) as described in the manufacturer's instructions, with the corresponding antibodies. Sample analysis was conducted using the Flow Cytometer Cytomics FC 500 (Beckman Coulter), and FlowJo software (BD Biosceince, V10.8.1) was used for data analysis.

2.7 RT-qPCR

16 spheroids were collected at each timepoint, and experiments were performed in triplicate (n=3). Both the RNeasy Plus Micro Kit and the RNeasy Plus Mini Kit from Qiagen were used for RNA isolation. 1500ng of the extracted RNA was used for reverse transcription using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Fisher Scientific), in accordance with the manufacturer's protocol. GAPDH was used as a reference gene. Gene-specific primers

were created using the Primer3 program (Suppl. Table 3). Each qPCR reaction contained a 5ng cDNA template, 50% SYBR Green JumpStart Taq ReadyMix, 400nM of each primer to a final volume of 15µl. The qPCR reaction was set up using the Rotor-Gene Q cycler (Qiagen) for qPCR reaction and the QIAgility liquid handling robot for experimental setup. The denaturation step of the qPCR cycling procedure was 3 min at 94 °C, followed by 40 cycles of 5 s at 95 °C, 15 s at 60 °C, and 30 s at 72 °C. Melting curve analysis was used to establish primer specificity. For normalization, human cortical RNA (Takara Bio, Cat# 636561) and foetal brain RNA (Takara Bio, Cat# 636526) were utilised. Data from three technical replicates for each gene were analysed using the ddCT technique (Livak & Schmittgen, 2001).

2.8 Cell viability and cytotoxicity testing

2.8.1 ATP Viability Assay

To generate dose-response curves, NSC spheroids were exposed to progressively higher doses of compounds for 48 hours or 72 hours. Three technical replicates of each concentration were employed in each experimental plate, and three separate assays (n=3) were performed to collect the results. The vehicle control was NIM with 0.1% DMSO added. As a positive neurotoxic control, paraquat was used. The ATP viability experiment was carried out using CellTiter-Glo® 3D Cell Viability experiment (Promega), in accordance with the manufacturer's instructions. Following BPA treatment, the NSC spheroids received 100µl of CellTiter-Glo® 3D Reagent for 1 hour at room temperature. The Thermo VarioScan Flash plate reader (Thermo Fisher Scientific) was then used to record the luminometric signal.

2.8.2 LDH Cytotoxicity Assay

As previously described, during the 21-day neural induction procedure, spheroids were exposed repeatedly to nano- and micromolar concentrations of BPA. At D14 and D21 of the BPA-treated neural induction, treated NIM was aspirated from triplicate wells for each BPA concentration, as well as the vehicle control, to investigate LDH release. Using negative controls on the respective days of the BPA treatment, cytotoxicity was determined on days 14 and 21 of the BPA-treated neural induction. Three independent experiments (n=3) were conducted. LDH levels from the collected medium were measured to estimate cytotoxicity using the CyQUANT™ LDH Cytotoxicity Assay (Thermo Fisher Scientific), according to the manufacturer's instructions. Colorimetric signal was measured using a Thermo VarioScan Flash plate reader.

2.9 Spheroid size analysis

Using the Olympus IX71 microscope and DP21 camera (DP21), images of BPA treated, and vehicle treated spheroids were captured. The CellSens Dimension program (version 1.11) was used to calculate surface area of the imaged spheroids. Eight spheroids at each concentration were measured in a total of four independent experiments (n=4), and each final value reflects the average of those measurements.

2.10 Quantitative proteomics

2.10.1 Sample preparation

Using a Sonopuls HD3200 (Bandelin, Berlin, Germany) cells were ultrasonically lysed in 8M urea/0.5M NH4HCO3 for 18 cycles of 10s. The Pierce 660nm Protein Assay (Thermo Fisher Scientific in Rockford, Illinois, USA) was used for protein quantification. 20µg of protein was reduced using 2 mM tris(2-carboxyethyl) phosphine (TCEP) and 4mM dithiothreitol (DTT)for 30 min at 56°C. Alkylation was then performed using 8mM iodoacetamide (IAA) in the dark at room temperature. To quench residual IAA, DTT was added to a final concentration of 10mM and incubated for 15 min in the dark. Protein digestion was then carried out using modified porcine trypsin (Promega, enzyme/protein ration 1:50) at 37°C for 16h.

2.10.2 Nano-liquid chromatography (LC)–tandem mass spectrometry (MS/MS) analysis and statistics

On an UltiMate 3000 nano-LC system connected online to a Q-Exactive HF-X instrument from Thermo Fisher Scientific, 1µg of the digest was injected. Peptides were first transferred to a PepMap 100 C18 trap column (100 m x 2 cm, 5 M particles, Thermo Fisher Scientific) before being separated on a PepMap RSLC C18 analytical column (75 m x 50 cm, 2 M particles, Thermo Fisher Scientific) at 250 nl/min flow rate with a gradient of 5-20% of solvent B for 80 minutes, followed by an increase to 40% for 9 minutes. Formic acid 0.1% in water made up solvent A, whereas formic acid 0.1% in acetonitrile made up solvent B. MS spectra were obtained utilizing one of the top 15 data-dependent acquisition methods. The dataset has been uploaded to the ProteomeXchange Consortium through the PRIDE partner repository using the dataset number PXD042045 (Perez-Riverol et al., 2022). MaxQuant (Tyanova et al., 2016) was used to process raw files using the human SwissProt reference proteome, which was downloaded in October 2022. Using customised R scripts, all statistical analysis and data visualizations were carried out. The MS-EmpiRe (Ammar et al., 2019) method, which was previously reported (Flenkenthaler et al., 2021) was used to test for differential abundance in proteins with at least two peptides found in at least three samples of each condition. Data imputation using random numbers from the normal distribution (downshift 1.8, width 0.8) was used to address missing values for peptides having measurements in all replicates of one condition but insufficient measurements in the other condition. Proteins were deemed significantly changed if their fold-change was greater than 1.3 and their Benjamini-Hochberg adjusted P-value was less than 0.05. ComplexHeatmap R package (Gu et al., 2016) was used for hierarchical clustering. The heatmap was divided into homogenous sections using the k-means method. The WebGestaltR software (Liao et al., 2019) and the functional category "GO Biological Process nonRedundant" were used to conduct the over-representation analysis. The Benjamini-Hochberg approach was used to regulate the false discovery rate.

2.11 Statistical analysis

Data is presented as the mean with the standard error of the mean (SEM). Prism 7 (Graphpad Software, CA, USA) software was used to analyse all data, with the exception of the proteomics

dataset. When appropriate, one-way ANOVA, two-way ANOVA, and Dunnett's post hoc test were used to establish statistical significance. P values under 0.05 indicated significance.

2.12 Network analysis

2.12.1 Human Protein-Protein Interaction (PPI) network construction and analysis

The human PPI network, which contains 18,816 proteins and 478,353 physical interactions, was constructed using publicly accessible resources (Alanis-Lobato et al., 2017; Luck et al., 2020; Menche et al., 2015). The connectivity of each group of proteins was determined by generating a z-score of the biggest linked component for each group of proteins in comparison to 10,000 randomly chosen protein sets of the same size. Significantly altered proteins were mapped onto the human PPI network. We took into account all proteins that were differently abundant for each condition, as well as breaking them down into up- and down-regulated proteins.

2.12.2 Enrichment analysis

By performing an enrichment analysis for the three major branches of the gene ontology (GO) (Ashburner et al., 2000): biological processes (BP), molecular functions (MF), and cellular components (CC), as well as for the KEGG pathway (Kanehisa & Goto, 2000) using GSEAPY (Fang et al., 2023), differently abundant proteins and their corresponding connected core were biologically characterised.

2.12.3 Disease predictions

DisGeNet (Piñero et al., 2015), the biggest publicly accessible database of genes and variations linked with human illnesses, was used to obtain diseases-gene associations (GDA). This collection includes associations that have been expertly selected from GWAS catalogues, animal models, and scientific literature. Each gene-disease connection is given a GDA score, which ranges from 0 to 1, based on the accuracy of the information. In order to retrieve data for 11,099 disorders, we only chose relationships having a GDA score >0.3. The association between each set of proteins with differential abundance (s1) and set of proteins associated with disease (s2) was then determined in two distinct ways: 1) by calculating their Jaccard index (intersection (s1,s2)/union(s1,s2)), and 2) by the closeness of the two sets' networks (Guney et al., 2016).

By comparing it to 10,000 randomly selected sets of topologically related proteins, Network proximity calculates the proximity of two sets of proteins in a network. In this method, biases in the interactome, such as the heavy-tail degree distribution and the discretization of other widely used network distances, including the shortest path, were taken into account and rectified. By considering both the shortest path and the presence of hubs in close proximity to the two gene sets, the shortest path between the differentially abundant proteins and the disease-related genes was calculated (source code is available at github.com/superlsd/NetBPAbrain).

3 RESULTS

3.1 Characterization of the 3D Neural Induction

To assess the suitability of the 3D neural induction to model NSC differentiation in vitro, we characterised hiPSC-derived NSCs. We measured the expression levels of key NSC transcripts throughout the duration of the neural induction using RT-qPCR. There were significant increases in the mRNA levels of classical neuroectodermal markers Sox1 and Nestin, as well as multipotency marker Sox2, by day 7 of the induction. Throughout the induction, we also observed a progressive elevation to the transcript levels of critical neural lineage proteins Tubulin-3 (Tub3), doublecortin (DCX) and Vimentin, as well as a decrease to marker of pluripotency Oct-4 by day 7 of the differentiation. We also examined proteome-wide changes in a comprehensive and unbiased manner. To do so, we used a label-free liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS) for the investigation of NSCs (day 21, $n = 5$) and hiPSCs (day 0, $n = 5$). 4733 proteins were identified. 60 % of the quantified were altered at day 21 compared to day 0. Typical NSC markers such as DACH1, MSI1, and MAP2 were amongst those with the highest increases of abundance at day 21, while other proteins of the neural lineage such as DCX, NCAM1 and FABP7 were also increased at day 21. Importantly, neural lineage proteins Tub3, Sox2, Vimentin and Nestin were all increased in abundance at day 21 compared to day 0, which is in accordance with our previous data. On the other hand, pluripotency markers LIN28A and Oct-4 were decreased in abundance in NSCs compared to hiPSCs.

3.2 Viability analysis of the BPA treated neural induction of hiPSCs

To determine sub-cytotoxic concentrations of BPA, we exposed hiPSC-derived NSC spheroids with increasing concentrations of BPA for 72-hours and performed a cell viability assay. A single dose of BPA at a concentration of 100µM for 72-hours significantly decreased the viability of NSCs by 33% compared to the vehicle-treated control group. Conversely, no significant alteration to NSC viability was detected in NSC spheroids treated with 0.01µM-50µM BPA for 72-hours.

After determining a range of BPA concentrations that were sub-cytotoxic and in-line with environmentally relevant exposure levels (E. Ribeiro et al., 2017, 2019; Zahra et al., 2022), we performed the repeated-dose BPA exposure during the neural induction of hiPSCs to NSCs using 0µM, 0.01µM, 0.1µM and 1µM BPA. Next, we utilised cytotoxicity assays to investigate whether the repeated-dose BPA exposure would impact cell survival. No significant alterations to cell viability (%) were detected at day 14 or day 21 of the BPA-treated neural induction, compared to vehicle-treated controls.

3.3 Repeated-dose BPA exposure affects NSC spheroid growth

To evaluate the effects that the repeated-dose BPA exposure exerted on NSC spheroid growth rate during the neural induction, we performed surface area measurements from brightfield images

throughout the differentiation. From day 3 to day 15, no significant alteration in spheroid size was detected between BPA-treated groups and the vehicle control group. Interestingly, at day 21 of the differentiation, there were significant decreases in spheroid size; 32%, 25%, and 27% in 0.01µM, 0.1µM and 1µM BPA treated groups, respectively.

3.4 Proteome alterations in BPA-treated NSCs

To assess the effects of BPA exposure on the proteome profile of NSCs in a comprehensive and unbiased manner, we utilised a label-free LC-MS/MS of day 21 NSCs that were exposed to repeated doses of 0µM (vehicle-treated control group, $n = 4$), 0.01µM ($n = 4$), 0.1µM ($n = 4$), 1µM ($n = 4$) BPA for the duration of the 3D neural induction. 11, 39 and 66 proteins were significantly changed in 0.01µM, 0.1µM and 1µM BPA-treated groups, respectively, when compared to the vehicle-treated control group. Notably, many proteins were significantly altered in common across all BPA-treated groups, for example, there were decreases to GAP43, TPPP3, Wnt-8b and GPC4. On the other hand, there were increases to FABP7 in all treated groups. The protein alterations in BPA treated NSCs showed a dose-dependent increase in the number of differentially changed proteins. Many of the altered proteins in all BPA-treated groups maintain vital roles in NSC maintenance and the development of the foetal brain.

3.5 Protein and disease network analysis of BPA-treated NSCs

To assess the molecular interactions and the functions of the differentially abundant proteins in disease, we mapped them in a human PPI network Because of the dose-dependency of the findings, we further investigated the down-regulated proteins in 0.1µM and 1µM BPA treated groups. Of the 18 down-regulated proteins in these groups, we identified 6 proteins (PGAM1, CKB, ALDOA, ENO1, ENO2 and TPI1) that closely interacted in the PPI (*p*-value: 1.2e-12). These proteins will be referred to as the BPA-downregulated core proteins, which were enriched for both the glycolytic pathway and HIF-1 signalling pathway. To investigate the possible links between the BPA downregulated core and brain-related diseases we examined its representation on a DisGeNET compilated list of over 11,000 diseases and calculated a Jaccard index which computed a pairwise combination of their genetic overlap. We found a positive association with acute schizophrenia and enzymopathy.

For further investigation of the disease associations of the BPA down-regulated core, we generated a network that encompassed these proteins in addition to their shortest links to a group of brain disorders such as Schizophrenia, ASD, Dementia, ALS, Depression and AD. Most of the BPA downregulated core proteins interacted with protein hubs that have previously been reported to play a role in many neurological conditions, such as ESR2 (Pinsonneault et al., 2013), and APP (Jakobsson et al., 2013; Sun et al., 2008).

We saw that the BPA-induced changes in ENO1 and ALS expression could lead to several brain disorders by regulating their direct interactors. An example of this is the interaction of ALDOA with GLO1 and HDAC which have previous links with depression, while additionally, SOD1 and

FUS also interact with ALDOA, and these proteins have causal roles in ALS (Deng et al., 2014; Miller et al., 2013).

To deepen this investigation, we assessed the transcriptomic expression levels of genes with causative roles in ALS, and that also interact with ENO1 and ALDOA in the largest presently available ALS cohort. From this cohort, we discovered that the expression of these genes was significantly lower in the cervical and lumbar regions of ALS patients when compared to controls.

To summarise, our results indicate that BPA induces a dose-dependent effect on neurodevelopmental processes by altering the number of differentially expressed proteins, and potentially, their interactions. This could lead to glycolytic metabolism impairments, which are known to underlying causes in several brain diseases.

4 NEW SCIENTIFIC RESULTS

In this research, we investigated the effects of a repeated-dose BPA exposure during the neural induction of hiPSCs to NSCs to model the effects of BPA on the earliest stages of embryonic brain development. HiPSC-derived NSCs were successfully established and characterised in detail, emphasizing the strength of this model for neurotoxicological screening applications as well as other DOHaD-related investigations. Following this, a broad range of cellular and molecular investigations were utilised to examine the effects that BPA exposure during neurulation exerts on NSCs in the developing brain, and their potential implications with regards to NDs and NDDs. The novel findings of this study include:

1. We postulated that observing patterns of vulnerability in culture-perturbed imprinting aberrations in hiPSCs could enhance the understanding of the effects of ART procedures on embryos, which was confirmed by the matching patterns of imprint vulnerability in hiPSCs and animal models of ART in literature.
2. We have produced a novel in vitro model for the investigation of the effects of repeated-dose exposures of environmental chemicals during the neural induction of hiPSCs to NSCs, representative of the neurulation stage of embryonic brain development.
3. We performed, for the first time, BPA treatment during the neural induction of hiPSCs to NSCs, offering a new perspective on the effects of BPA on the earliest stage of brain development.
4. For the first time, we performed a proteomics-based analysis on BPA-treated NSCs uncovering novel molecular alterations, and, via network analysis, identified links between BPA-perturbed proteins and several NDDs and NDs in a human cell-based model. Of note, we identified a potential pathological association between BPA perturbed proteins (ALDOA and ENO1) and several causal genes in ALS via protein interactome analysis.
5. In this study, we observed BPA-induced Wnt alterations for the first time in a human cell-based model.
6. We demonstrated alterations to the glycolytic signalling pathway in BPA-treated human NSCs for the first time.

5 DISCUSSION AND FUTURE PERSPECTIVES

In the present study, we critically evaluated the capacity of hiPSC-based models in literature for investigations into ART-associated IDs. Promisingly, hiPSC-derived systems can recapture the aberrant imprint patterns observed in ID patients, which enables the study of the effects of ART-associated IDs, including PWS, SRS and AS on differential processes during brain development. Moreover, we also highlighted evidence form previous studies showing that culture perturbed imprints in reprogrammed hiPSCs show similar patterns of susceptibility as animal models of ART. Therefore, enhancing our understanding of how reprogramming procedures and in vitro culture affects imprinting regulation in hiPSCs can also elucidate our understanding of the association between ART procedures and IDs.

The methylation changes and LOI described in normal hiPSCs upon reprogramming reflect several identical aberrations observed in embryos after ART. Improving the understanding of the precise mechanisms responsible for the loss of regulation of genomic imprinting in hiPSCs can also clarify the underlying epigenetic mechanisms in IDs and how they are altered by various ART procedures. Genomic imprinting was discovered many years after the first successful ART conception; this is a potent example of how emerging medical technologies frequently surpass our fundamental understanding of the associated biological processes. Additionally, this emphasises the significance of regularly bettering and reevaluating established methods. The knowledge obtained from stem cell and animal-based studies can aid in improving the safety and reducing epigenetic errors induced by ART procedures, leading to more promising health outcomes for ART patients.

Moving forward, large cohort studies with comprehensive and standardised methods of analysis of ART populations are needed, however, for the time being, model systems are the benchmark for enhancing our understanding of how ART procedures affect the epigenome. Mouse models enabled several pioneering discoveries regarding genomic imprinting and maintain a critical role in clarifying the association between IDs and ART procedures. However, departures in preimplantation development and the regulation of genomic imprinting between humans and rodents highlight the requirement for human studies into imprinting and IDs. Utilizing human pluripotent stem cells bridges the gap between animal models and clinical data.

Many groups have studied the effects of BPA exposure on NSCs and NPCs, including alterations to their proliferative capacity, with contradicting results depending on the duration of exposure and the concentration of BPA used. Currently, there is a paucity of data regarding BPA's effects on the developing human brain since present knowledge was primarily derived from animal studies. In this study, we demonstrated that our 3D in vitro model was robust and effective for the differentiation of hiPSCs to NSCs. We observed increases in the expression of key NSC and neuroectodermal markers including Sox2 and Nestin during the 3D neural induction on both an mRNA and protein level using ICC visualization and RT-qPCR. Furthermore, we utilised MS/MS-based proteomics, which complemented our previous data showing increases to several key NSC and neural lineage proteins by day 21 of the 3D neural induction (Červenka et al., 2021; Galiakberova & Dashinimaev, 2020; Shin et al., 2007; Yun et al., 2012). As the system utilised in the study was a human cell-based model, we therefore provide a NAM that is capable for producing, translatable, human relevant data for toxicity during the earliest phases of embryonic brain development. Our protocol can facilitate the investigation of the effects of environmental

chemicals on the NSC and neuronal stages of differentiation on both a cellular and molecular level, and can also be used to differentiate ID patient-derived hiPSCs to investigate the pathological effects that faulty imprints impose on the developing embryonic brain.

In the present study, we investigated the effects of nM to µM (0.01µM - 1µM) BPA concentrations in-line with realistic environmental concentration levels (Ribeiro et al., 2017, 2019; Zahra et al., 2022). This enabled us to model the effects of a repeated environmental exposure to BPA, replicating a real-life scenario in an in vitro setting. The 21-day protocol for the BPA-treated 3D neural induction, for the first time, enabled the examination of the effects that BPA induces on the neural induction of hiPSCs. Additionally, we demonstrated a reduction in the size of spheroids in each BPA-treated group at day 21, suggesting that BPA inhibited NSC proliferation. Our results complement data from previous studies showing that BPA exposure reduced NSC and NPC proliferation (Huang et al., 2019; Kim et al., 2007; Rebolledo-Solleiro et al., 2021; Tiwari et al., 2015). Since no alterations to NSC viability were detected at day 21 of the BPA treated neural induction to accompany the reduction to spheroid size, we postulated that molecular alterations were implicated in the described changes.

To examine the molecular alterations induced during the BPA-treated neural induction, we investigated proteome-wide changes in BPA-treated NSCs. A proteomic analysis showed that many proteins with critical functions in NSC maintenance, proliferation and differentiation were differentially abundant 0.01µM, 0.1µM and 1µM BPA-treated groups. We observed a decrease to canonical Wnt-signalling pathway protein Wnt-8b in all treated groups. Notably, Wnt-8b maintains an important role in neuroectodermal patterning, while its expression is also associated with the neural tube stage of development (Ciani & Salinas, 2005; Kim et al., 2002). Therefore, the reduced level of Wnt-8b induced by BPA exposure could impact the regional pattern specification of NSCs during embryonic brain development. Our observations of decreased Wnt-8b protein in BPA-treated groups are complemented by prior studies using rat models that detected errors to the Wnt signalling pathway that led to the inhibition of NSC proliferation (Tiwari et al., 2015, 2016). Furthermore, this study was the first to confirm BPA-induced canonical Wnt alterations in a human NSC model.

Additionally, we detected a decrease to Neuromodulin (GAP43) in every BPA-treated group. GAP43 is a protein specifically found in the nervous system and maintains a critical role in mitotic NSCs and NPCs (Brittis et al., 1995; Esdar et al., 1999). GAP43 expression has been shown to be enriched in proliferating areas of the embryonic brain, and interestingly, a loss of GAP43 expression leads to an inhibition of NPC proliferation (Kanazir et al., 1996; Mani et al., 2001; Mishra et al., 2008). The proteomics analysis also detected decreases to TPPP3 (p20) inclusively in all BPA-treated groups. Inhibiting or knocking-down p20 has previously been shown to result in cell cycle arrest and inhibited proliferation in several mouse and human tumour cell lines which suggests that the BPA-induced alteration to p20 in our study could be implicated in the reported changes to NSC proliferation. Glypican-4 (GPC4), a protein typically expressed by NSCs of the ventricular zone in the developing brain, was also decreased in all BPA-treated groups. GPC4 expression usually decreases during NSC commitment (Hagihara et al., 2000), while the downregulation of GPC4 in NSCs has been shown to affect NSC maintenance and proliferation. Interestingly, the downregulation of GPC4 in NSCs pushes the balance from the maintenance of the NSC pool towards NPC differentiation (Fico et al., 2012), resulting in an untimely deficiency of NSCs. Therefore, the reported decrease to GPC4 in the current study complements the

mechanistic evidence from prior studies showing that BPA-induced changes to the Wnt signalling cascade led to suppressed NSC proliferation (Tiwari et al., 2015, 2016).

Our findings demonstrated that NSCs exposed to 0.1μM and 1μM BPA during the neural induction showed potential aberrations to the glycolytic pathway. Proliferating NSCs are dependent upon glycolysis rather than oxidative phosphorylation (OXPHOS) as the main source of ATP synthesis (Iwata & Vanderhaeghen, 2021; Zheng et al., 2016). The initiation of neurogenesis begins with NSC proliferation and gradual transition to NPCs, which is followed by neuronal differentiation. During this process, there is a continuous metabolic conversion; in unison with the differentiation of NSCs to NPCs and neurons, the primary mechanism for ATP synthesis transitions from glycolysis to OXPHOS (Iwata & Vanderhaeghen, 2021; Maffezzini et al., 2020; Zheng et al., 2016). Thus, decreased abundances to proteins involved in the glycolytic pathway, could complement the assertion that NSCs exposed to BPA throughout the neural induction are at a more 'primed' NPC phase as glycolytic processes decrease and their differentiation advances. What remains unclear is whether the proliferation impairments caused by BPA exposure are a cause or an effect of decreases to key proteins of the glycolytic pathway.

In this study, we demonstrated that BPA exposure resulted in decreases to several glycolytic proteins in NSCs, with the potential to lead to neurological defects during embryonic brain development and beyond that could result in the onset of NDs and NDDs. For instance, we demonstrated that ALDOA and ENO1 interact with MED13, which has been linked to several NDDs such as intellectual disability, ASD, and encephalopathy (De Nardi et al., 2021; Trivisano et al., 2022). We also showed that impairments to the glycolytic metabolism could contribute to ALS onset via the interactions of ALDOA with ESR2, FUS and SOD1. Intriguingly, the decreased expression of APP, SPG7 and ESR2, which directly interact with ENO1 and ALDOA of the glycolytic signalling pathway, was also observed in the lumbar and cervical regions in an independent ALS cohort. Even though there remains, at present, a lack of evidence directly linking ALS with BPA exposure, there is a possibility that BPA-induced metabolic or physiological disruptions, including disturbances to glycolysis, could play a part in the development of ALS. For example, many pre-clinical studies have shown that aberrations to glycolytic metabolism and transport in the CNS could promote the progression of ALS (Tefera et al., 2021), and recent findings from studies using mouse models have described glycolytic disturbances in ALS (Ferraiuolo et al., 2011; Tefera et al., 2019; Tefera & Borges, 2019).

In conclusion, we present a novel NAM for investigating the effects of environmental chemical exposures and IDs on NSC differentiation during the earliest developmental phase of the CNS. We showed that repeated-doses of BPA over a 21-day period with environmentally relevant, sub-cytotoxic concentrations of BPA induced a decreased spheroid size during the hiPSC to NSC neural induction, which is in support of previous studies showing that BPA suppressed NSC proliferation. We also uncovered BPA-induced changes to the expression of several proteins with critical roles in the proliferative and differentiative processes of NSCs, as well as the glycolytic pathway, with connotations for our current understanding of BPA's effects on the maintenance and differentiation of NSCs during the PC stages of brain development. Aligned with the DOHaD theory, we provide mechanistic information into the association between several brain disorders and BPA exposure during embryonic CNS development, suggesting that BPA exposure leads to glycolytic alterations that can ultimately play a role in the pathophysiology of NDs, such as ALS, as well as NDDs including ASD and intellectual disability.

6 PUBLICATIONS

International article publications:

- **Horánszky A**, Becker JL, Zana M, Ferguson-Smith AC, Dinnyés A. Epigenetic Mechanisms of ART-Related Imprinting Disorders: Lessons From iPSC and Mouse Models. Genes (Basel). 2021 Oct 26;12(11):1704. doi: 10.3390/genes12111704. PMID: 34828310; PMCID: PMC8620286.
- **Horánszky A**, Shashikadze B, Elkhateib R, Lombardo SD, Lamberto F, Zana M, Menche J, Fröhlich T, Dinnyés A. Proteomics and disease network associations evaluation of environmentally relevant Bisphenol A concentrations in a human 3D neural stem cell model. Front Cell Dev Biol. 2023 Aug 16;11:1236243. doi:10.3389/fcell.2023.1236243. PMID: 37664457; PMCID: PMC10472293.
- Rubini E, Baijens IM, **Horánszky A**, Schoenmakers S, Sinclair KD, Zana M, Dinnyés A, Steegers-Theunissen RPM, Rousian M. Maternal One-Carbon Metabolism during the Periconceptional Period and Human Foetal Brain Growth: A Systematic Review. Genes (Basel). 2021 Oct 17;12(10):1634. doi: 10.3390/genes12101634. PMID: 34681028; PMCID: PMC8535925.
- Mitrečić D, Hribljan V, Jagečić D, Isaković J, Lamberto F, **Horánszky A**, Zana M, Foldes G, Zavan B, Pivoriūnas A, Martinez S, Mazzini L, Radenovic L, Milasin J, Chachques JC, Buzanska L, Song MS, Dinnyés A. Regenerative Neurology and Regenerative Cardiology: Shared Hurdles and Achievements. Int J Mol Sci. 2022 Jan 13;23(2):855. doi: 10.3390/ijms23020855. PMID: 35055039; PMCID: PMC8776151.
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International poster and oral presentations:

- **Horánszky A**, Zana M, Dinnyés A. The impact of environmental exposures on the neuronal differentiation of pluripotent stem cells. IBRO 2022, Budapest, Hungary. January 2022.
- **Horánszky A**, Zana M, Dinnyés A. The impact of environmental exposures on the neuronal differentiation of pluripotent stem cells. Visegrad Group Society For Developmental Biology. Szeged, Hungary. September 2021
- **Horánszky A**, Zana M, Dinnyés A. The effects of Bisphenol A exposure on the neuronal differentiation of hiPSCs. RNA Symposium 2022. Vienna Biocenter, Austria.
- **Horánszky A**, Zana M, Dinnyés A. The effects of Bisphenol A exposure on the neural induction of hiPSCs. FIBOK 2022, MATE University, Gödöllő, Hungary. April 2022.

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