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

Doctoral (PhD) dissertation

Alex Horánszky

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Name: Doctoral School of Animal Biotechnology and Animal Science

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Leader of the school: Professor Dr Miklós Mézes, D.V.M., Member of the HAS

Head of Department,

Hungarian University of Agriculture and Life Sciences, Institute of Physiology and Animal Nutrition, Department of Nutritional Safety

Supervisor: Professor Dr András Dinnyés, D.V.M, D.Sc.

Hungarian University of Agriculture and Life Sciences, Institute of Physiology and Animal Nutrition, Department of Physiology and Animal Health

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Approval of the PhD School leader

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Approval of the Supervisor

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LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
AD	Alzheimer's disease
ADHD	Attention deficit hyperactivity disorder
ALS	Amyotrophic lateral sclerosis
ART	Assisted reproductive technologies
AS	Angelman syndrome
ASD	Autism spectrum disorder
AR	Androgen receptor
Arh	Aryl hydrocarbon receptor
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
bFGF	Basic fibroblast growth factor
BPA	Bisphenol A
BWS	Beckwith-Wiedemann syndrome
CDK4	Cyclin-dependent kinase 4
CNS	Central nervous system
CREB	cAMP response-element binding
DAPI	4′,6-diamidino-2-phenylindole
DCX	Doublecortin
DMR	Differentially methylated region
Dmrta1	Doublesex- And Mab-3-Related Transcription Factor A1
DMSO	Dimethyl Sulfoxide
DNMT	DNA methyltransferase
DNT	Developmental neurotoxicity
DOHaD	Developmental origins of health and disease
DTT	Dithiothreitol
Dbn	Drebin
EDC	Endocrine disrupting compound
EGF	Epidermal growth factor
ER	Estrogen receptor

ERR	Estrogen related receptor
ESC	Embryonic stem cell
GAP43	Neuromodulin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GI	Gastrointestinal
GOM	Gain of methylation
GPC4	Glypican-4
GR	Glucocorticoid receptors
Hes1	Hairy and enhancer of split-1
hiPSC	Human induced pluripotent stem cell
HPA	Hypothalamic-pituitary-adrenal
IAA	Iodoacetamide
IC	Imprinting center
ICR	Imprinting control region
ICSI	Intracytoplasmic sperm injection
IDs	Imprinting disorders
IVF	In vitro fertilization
JNK	The c-Jun N-terminal kinase
KAP1	KRAB-associated protein 1
LDH	Lactate dehydrogenase
lncRNA	Long non-coding RNA
LOI	Loss of imprinting
LOM	Loss of methylation
MAP2	Microtubule associated protein 2
MIE	Molecular initiating event
mRNA	Messenger ribonucleic acid
NAM	New approach methodology
NCD	Non-communicable disease
ND	Neurodegenerative disease
NDD	Neurodevelopmental disorder
NIM	Neural induction medium
NMM	Neural maintenance medium

NPC	Neural progenitor cell
NSC	Neural stem cell
ntESC	Nuclear transfer embryonic stem cell
Oct4	Octamer-binding transcription factor 4
OXPHOS	Oxidative phoshorylation
P53	Tumour protein 53
PAX6	Paired box protein pax-6
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PFA	Paraformaldehyde
PNS	Peripheral nervous system
PPI	Protein-protein interaction
PWS	Prader-Willi syndrome
RC2	Radial-glial cell marker-2
ROS	Reactive oxygen species
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SMAD	Mothers against decapentaplegic
SOX2	SRY-box transcription factor 2
SRS	Silver-Russell syndrome
SYP	Synaptophysin
Tau	Tubulin associated unit
TCEP	Tris(2-carboxyethyl) phosphine
TET	Ten-eleven translocation methylcytosine dioxygenase
TGF-β	Transforming growth factor beta
P20	Tubulin polymerization promoting protein family member 3 (TPPP3)
TR	Thyroid hormone receptor
TUBB3	Beta 3 tubulin
USD	Uniparental disomy
ZNF57	Zinc finger protein 57 homolog
ZNF445	Zinc finger protein 445

1 INTRODUCTION

The periconceptional period of development represents a 5- to 6-month time window encompassing oocyte growth, fertilization, gamete maturation and embryonic development up until week 10 of gestation (Steegers-Theunissen et al., 2013). Approximately 15-20% of pregnancies end with spontaneous pregnancy loss during the first 24 weeks, for as-of-yet unexplained reasons (X. Wang et al., 2003). There is strong evidence demonstrating that environmental alterations during the periconceptional period elicit long-term health effects in offspring; currently, data from both animal models and human epidemiological studies show that children display conditions and potential disease risks that are associated with parental exposures (Chan et al., 2015), which could also contribute to spontaneous pregnancy loss. Such exposures include but are not limited to chemical stressors during pregnancy and assisted reproductive technologies (ART) (Heindel et al., 2017; Horánszky et al., 2021).

The Developmental Origins of Health and Disease (DOHaD) concept associates altered environmental conditions during the periconceptional period, such as those previously mentioned, to an individual's health and metabolic condition throughout their lifetime. The periconceptional period is a particularly vulnerable stage in development, in which environmental alterations can induce aberrant phenotypic alterations from both direct stress and adaptive compensatory mechanisms, resulting in aberrant phenotypic changes later in life (Barker, 2004). Currently there is a global increase in the prevalence of non-communicable diseases (NCDs), including neurological disorders, affecting millions of people annually (Feigin et al., 2020; Sirenko et al., 2019). The observed increase in NCDs and has been attributed, at least in part, to altered environmental conditions, such as chemical exposures during the periconceptional stages of development (Fleming et al., 2018; Grandjean & Landrigan, 2006). To date, the mechanisms underlying the cellular, metabolic, and physiological changes that lead to NCDs are unclear, and require further elucidation. Moreover, there remains an urgent requirement to determine the contributing factors of NCDs, as current evidence supporting the DOHaD hypothesis suggests that early-life environmental interventions, including during the periconceptional period, may prove decisive in the prevention of DOHaD-associated NCDs (Bay et al., 2019). Thus, the increasing prevalence of NCDs is accompanied by an urgent need for human relevant models that can provide translatable information to assist in our understanding of the risk factors and underlying causes of these diseases.

The development of the brain involves sophisticated interactions between both environmental and genetic factors. Environmental disturbances to the brain during vulnerable developmental stages

can lead to aberrations to brain structure, connectivity, and function, with substantial consequences (Money & Stanwood, 2013). The susceptibility of the fetal brain to environmental insults is evident, and exposure to environmental chemicals can result in the disruption of the earliest processes in embryonic brain development such as neurulation, neuronal proliferation and differentiation, as well as subsequent processes including synaptogenesis, dendritic outgrowth and myelination (Chesnut et al., 2021; Guo et al., 2013; Hornung et al., 2009; Huang et al., 2019; Kalloo et al., 2021; Ling et al., 2016; Schneider et al., 2003). Increasing evidence suggests that the potential adverse neurological outcomes that can arise due to environmental challenges during the fetal 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) (Doi et al., 2022; Modgil et al., 2014; VAN DEN BERGH, 2011).

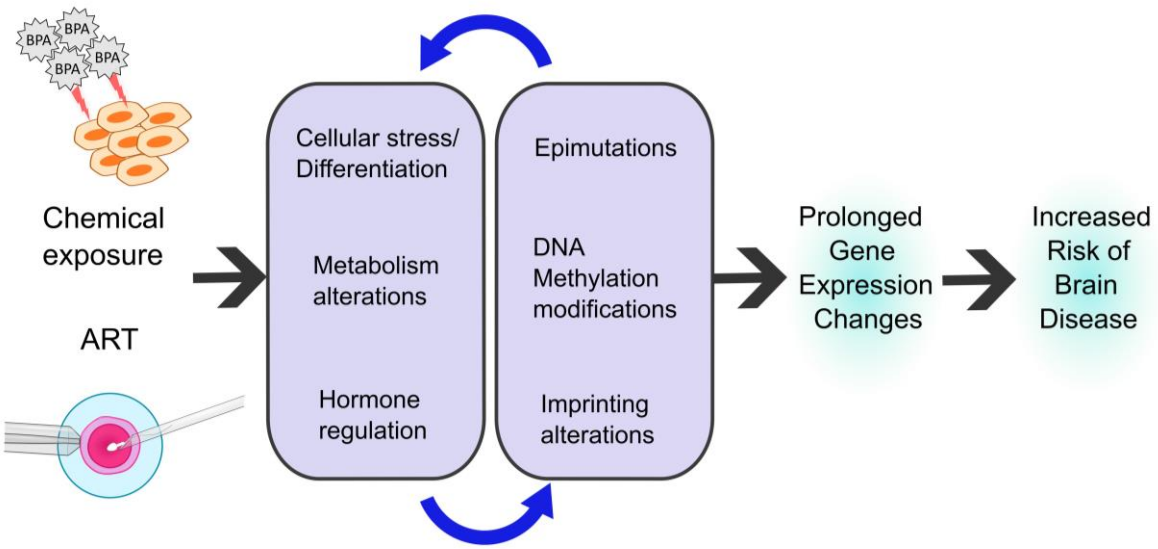

Figure 1. Both the exposure to environmental chemical chemicals during development and ART procedures are associated with elevated risk of NCDs. The DOHaD hypothesis states that developmental challenges such as these can contribute to the onset of brain disorders throughout an individual's lifetime.

Epigenetic mechanisms have been proposed to provide a further mechanistic explanation between DOHaD-related environmental exposures and phenotypic alterations. Epigenetics concerns the study of the nuclear chromatin landscape which is modulated via epigenetic marks such as DNA methylation and post-translational histone modifications (Safi-Stibler & Gabory, 2020; Yamada & Chong, 2017). It is now widely accepted that changes to epigenetic signatures are important to explain alterations to long-term health programming induced by early-life environmental exposures, while they also aid in the explanation of the underlying molecular alterations that could

eventually be used for predicting or preventing NCDs (Yamada & Chong, 2017). Most evidence in support of the DOHaD concept was obtained from animal models and human observational studies (Fleming et al., 2018; McMullen & Mostyn, 2009). To date, human based DOHaD investigations have relied upon children conceived via ART procedures; an ever-growing clinical cohort that includes millions of people globally (Fleming et al., 2018). 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 (Feuer & Rinaudo, 2016; Hansen et al., 2016; Sunde et al., 2016). 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 (Lazaraviciute et al., 2014). Moreover, a growing number of studies suggest an association between ART and imprinting disorders (IDs), including the neurodevelopmental disorders Angelman syndrome (AS) and Prader-Willi syndrome (PWS), as well as Silver-Russell syndrome (SRS), which also induces aberrations to neurodevelopment (Horánszky et al., 2021; Isles, 2022; E. H. P. Ribeiro et al., 2022). 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 Angelman syndrome, Prader-Wili syndrome and Silver-Russell syndrome, which all have detrimental effects on the developing brain.

Epidemiological study has contributed important evidence that supports the DOHaD hypothesis (Chavatte-Palmer et al., 2016; Hoffman et al., 2017). It is also important to recognise the wealth of evidence produced by animal studies that has illustrated the endocrine, metabolic, and epigenetic mechanisms responsible for DOHaD-associated phenotype alterations. Furthermore, animal studies enhanced the understanding of the ‘plasticity’ of developing organisms, which is a direct consequence of their heightened susceptibility during critical developmental stages, such as the periconceptional period. The underlying mechanisms implicated in chemical exposure-induced aberrations observed in epidemiological studies has also been clarified using animal models (Dickinson et al., 2016; Heindel, 2019). The vital contributions of these models to the DOHaD field cannot be understated, however, in vitro cellular models are crucial for the advancement of the current knowledge regarding the DOHaD field. In vitro models allow for the detailed investigation tissue functionality and mechanistic pathways at various developmental time-points, while they also decrease the need for live animal models, overcoming the associated ethical complications.

Bisphenol A (BPA) is an endocrine disrupting compound 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 (Porras et al., 2014; Vandenberg et al., 2007). 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 (Hartle et al., 2022). Current reports suggest that human exposure to BPA is linked to behavioural alterations and impaired cognitive function, and additionally, to neurodevelopmental disorders such as schizophrenia and ASD, and neurodegenerative diseases including AD (Brown, 2009; Rebolledo-Solleiro et al., 2021). 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 (Agarwal et al., 2016; Dong et al., 2021; Gill & Kumara, 2021; Huang et al., 2019; K. Kim et al., 2007; Tiwari et al., 2015). 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 neurodevelopmental disorders and neurodegenerative diseases.

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 (Shi et al., 2017). hiPSC technology is a source of autologous, patient-specific cells that also bypass the ethical issues associated with human embryonic stem cells (ESCs) (Shi et al., 2017; K. Takahashi & Yamanaka, 2006). hiPSCs can be differentiated into practically any cell type and are now commonly used for the in vitro differentiation of specialised cells, including neural stem cells (NSCs) and neurons. The neural differentiation of hiPSCs can be utilised to study the impact of DOHaD-related chemical exposures on the developing human CNS, while they are also useful tools to further understand the association between ART procedures and IDs, providing human-relevant, translatable data to bridge the gap between human clinical observations and animal studies (Horánszky et al., 2021; J. Xie et al., 2020).

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. There are currently several differentiation protocols for obtaining neural cultures from hiPSCs which enable the generation of all neural cell types with the appropriate functional and physiological attributes required for disease modelling or neurotoxicological screening of environmental chemicals (Kobolak et al., 2020; L. Li et al., 2018). 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 (Chambers et al., 2009; Galiakberova & Dashinimaev, 2020).

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 model can be used to assess neurodevelopmental toxicity of DOHaD-related environmental chemical exposures during the neurulation stages of brain development. 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. Our human cell-based model permitted further study into the disturbances caused by environmentally relevant BPA concentrations during the early phases of embryonic brain development and supported earlier findings from animal models. 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 neurodevelopmental disorders and neurodegenerative diseases.

1.1 Objectives of the PhD

In this study, firstly we critically reviewed the use of hiPSCs for modelling ART-induced imprinting disorders, and highlighted vulnerable imprints in the genome by comparing patterns of imprint and methylation defects in cultured hiPSCs and animal models of ART. The overall aim of the critical review was to clarify the association between ART procedures and IDs and the underlying epigenetic mechanisms.

Secondly, for the first time, we aimed to investigate the effects of a repeated-dose exposure of BPA during the in vitro 3D neural induction of hiPSCs to NSCs, emulating a chronic exposure during the neurulation stages of embryonic brain development.

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 LITERATURE OVERVIEW

2.1 Genomic imprinting & ART

Genomic imprinting is an epigenetic process that modulates the expression of certain genes in a parent-of-origin specific manner. Imprinted genes are exclusively expressed by either the maternal or paternal allele, with the unexpressed allele being epigenetically silenced (Ferguson-Smith, 2011). Imprinting control regions (ICRs) are *Cis*-acting regulatory elements that control imprinting in one or more genes. ICRs are methylated during male and female germline development by de novo methyltransferases in a parent-specific manner. These epigenetic marks are resistant to the epigenetic reprogramming that occurs post-fertilization, and therefore function as a memory of the parent of origin (Bartolomei & Ferguson-Smith, 2011). Therefore, differential methylation established in the germline is maintained through fertilization, leading to the appropriate dosage of imprinted genes via monoallelic gene expression. Imprinted regulation of gene expression is critical for resource allocation in the placenta and fetal growth, and additionally, in brain function, behaviour, and postnatal energy homeostasis (Davies et al., 2015; Tucci et al., 2019; Wilkinson et al., 2007). Thus, pre-natal development and post-natal health rely on the appropriate establishment and maintenance of the epigenetic signatures of imprinted genes. A typical example of imprinted gene regulation can be observed at the H19/IGF2 and CDKN1C/KCNQ1 imprinted loci (Figure 2). Epigenetic errors at this imprinted cluster can lead to the ID Beckwith Wiedemann syndrome (BWS) and several types of cancer (Lim et al., 2008; Tunster et al., 2011).

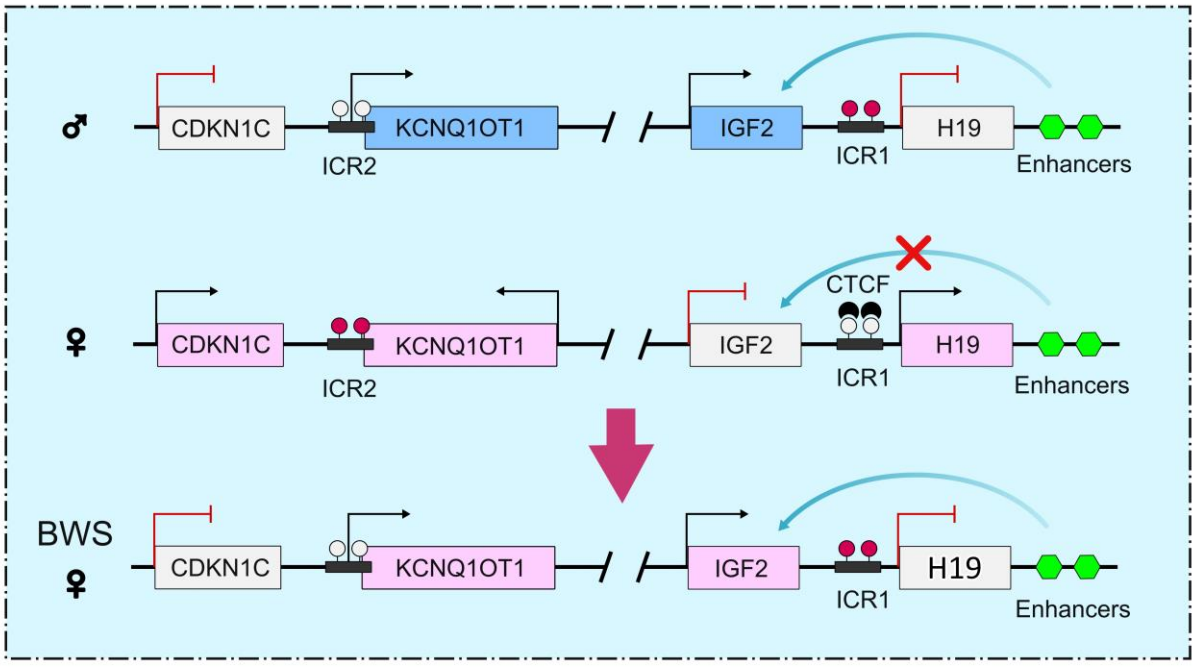

Figure 2. Schematic of the imprinting at the H19/IGF2 and the CDKN1C/KCNQ1 loci. On the maternal allele ICR2 is methylated while ICR1 remains unmethylated, resulting in the maternal expression of H19, KCNQ1 and CDKN1C and maternal silencing of IGF2 and KCNQ1OT1. Contrastingly, ICR2 remains unmethylated and ICR1 is methylated on the paternal allele, leading to the paternal expression of IGF2 and KCNQ1OT1. Epigenetic alterations such as uniparental disomy 11 and various epimutations at ICR1/ICR2 can lead to the onset of Beckwith Wiedemann syndrome where the maternal allele replicates the paternal allele resulting in the loss of expression of CDKN1C and the overexpression of IGF2.

Studies involving patients with IDs have clarified the influence of several imprinted genes. However, many IDs share similar phenotypes, rendering it difficult to provide an accurate diagnosis (Soellner et al., 2017). ID patients commonly exhibit faulty pre- and post-natal development, cognitive impairments, hormonal imbalances and potentially, poor feeding behaviour. The molecular aberrations associated to IDs include epimutations, genetic mutations, uniparental disomy (UPD), and copy number variation, and importantly, the same imprinted locus can be implicated in different IDs (Horánszky et al., 2021).

Manipulations of critical stages of conception are required to carry out ART procedures including ex vivo embryonic cultures, frozen embryo transfer, IVF, intracytoplasmic sperm injection (ICSI), and superovulation. Many of these processes have the potential to cause genomic imprinting aberrations (Chi et al., 2020). ART procedures are undertaken during sensitive stages of development during which a tightly regulated reprogramming of the epigenome occurs. This epigenetic reprogramming is particularly vulnerable to errors from environmental insults (Chi et al., 2020). Recently, there have been many investigations into the ramifications of ART on gametes and embryos. The ability to effectively determine the effects of ART on DNA methylation and genomic imprinting is hindered by differences between imprinted regions, the range of tissues and techniques utilised for measurements, and the heterogeneity of various ART procedures (Lazaraviciute et al., 2014). Further investigations are required to fully comprehend the extent of ART-related imprinting alterations in humans, with current knowledge restricted by ethical implications and a lack of proper models. To date, much data from animal models supports the ART-related implications to genomic imprinting. Due to the ever-increasing frequency of ART-facilitated births, further study is required to clarify the association between epigenetic changes, aberrant imprinting, and ART procedures. Standard measurement practices as well as larger and more clinically defined cohorts are required if these challenges are to be overcome. Despite their limitations, mouse models of ART have provided a solution to several of these obstacles, while hiPSCs also offer an exciting avenue to further understand the effects of ART procedures on DNA methylation and genomic imprinting.

2.1.1 ART-associated IDs

Increasing evidence suggests that there is an association between ART and IDs. Several reports investigating distinct cohorts have described increased rates of AS, SRS, and PWS in individuals conceived using ART procedures (Amor & Halliday, 2008; Cortessis et al., 2018; Eroglu & Layman, 2012; Henningsen et al., 2020; Manipalviratn et al., 2009; Odom & Segars, 2010). ART could potentially lead to aberrations to imprint establishment and maintenance; however, currently available data are not sufficient to draw reliable conclusions. Several important factors including maternal age, infertility and which ART method was used are rarely considered, while human ART patient studies do not investigate the underlying molecular alterations. As the number of global ART-conceived births continues to rise, surpassing a total of 10 million as of 2023 (Pinborg et al., 2023), so to the need to enhance the understanding of the ramifications of ART procedures on gametes and embryos.

A further understanding of the molecular defects implicated in IDs in ART populations could uncover patterns of susceptibility linked to ART. If specific aberrations are commonly observed in ART populations, it opens the possibility to highlight vulnerabilities that could assist in the improvement of ART procedures while simultaneously furthering our knowledge of the susceptibility of imprints to environmental alterations.

2.1.1.1 Prader-Willi Syndrome

Prader-Willi Syndrome (PWS) is an NDD, and patients display a range of symptoms including intellectual disability, developmental delay, restricted growth and hyperphagia. PWS patients also exhibit complex behavioural alterations with approximately 25% meeting the criteria for ASD (Fountain & Schaaf, 2016). The imprinted genes affected in PWS are *SNURF-SNRPN*, *MAGEL2*, *NECDIN*, and *MKRN3*. The molecular pathologies underlying PWS include microdeletions of the paternal copy of chromosome 15, UPD of the maternal allele, and epimutations (Cassidy et al., 2012). In the naturally conceived population, PWS prevalence lies between 1:10,000 to 1:30,000 births (DeAngelis et al., 2018). Previous studies have reported conflicting evidence regarding the association between ART procedures and PWS; studies investigating PWS cohorts in Finland, the USA and Denmark found no significant change to the incidence of PWS in ART populations (Gold et al., 2014; Henningsen et al., 2020). Conversely, a Japanese epidemiological study reported a link between PWS and ART (Hattori et al., 2019). Importantly, the studies that show no increase to PWS prevalence in ART populations can still provide valuable mechanistic information

regarding IDs and ART. For example, one study demonstrated that in PWS-ART patients, there was an increase in methylation errors and maternal UPD (Gold et al., 2014), demonstrating that ART procedures could inflict epigenetic aberrations whether they are directly associated with ART or not. Comprehensive epigenetic profiling of individuals conceived using ART procedures, in particular ID patients, will enhance our knowledge of the extent to which ART can alter DNA modifications.

2.1.1.2 Angelman syndrome

AS is a NDD typically characterised by seizures, speech impediments, intellectual disability and altered behaviours (Clayton-Smith, 2003), with a prevalence of approximately 1:10,000 to 1:20,000 people (Gentile et al., 2010). *UBE3A* is a maternally expressed gene critical during brain development. AS always involves alterations to *UBE3A* specifically, with around 65-75% of cases arising due to maternal chromosome microdeletions (Williams et al., 2010). Currently, it is unclear whether ART procedures are directly linked to AS, however, modelling AS can further the understanding of imprint mechanisms, especially when considering that the chromosomal errors leading to AS are well-defined.

2.1.1.3 Silver-Russell syndrome

The symptoms associated with SRS include low birth weight, intrauterine growth restriction, body asymmetry and slow postnatal growth. Additionally, SRS patients have difficulties with speech and in the global motor area, while certain subgroups exhibit mild learning difficulties and ASD symptoms (E. H. P. Ribeiro et al., 2022). The prevalence of SRS in the general population is between 1:30,000 to 1:100,000 (Wakeling et al., 2017) yet there remains no reliable approximation for SRS prevalence in ART-facilitated births. Achieving an accurate SRS diagnosis can be problematic, firstly, because the molecular basis of the disease is only established in approximately 60% of cases, and secondly, because patients often exhibit aspecific symptoms (Wakeling et al., 2010). The most frequently reported pathological feature of SRS is a loss of methylation (LOM) at the *H19/IGF2:IG*-differentially methylated region (DMR) at Ch11p15.5 (Wakeling et al., 2010). Also, in 5-10% of SRS patients, maternal UPD is observed on chromosome 7 (Abu-Amero et al., 2007). Previously, Japanese epidemiological studies observed the presence of DNA methylation errors in all ART-SRS cases in, while non-ART populations presented a normal distribution of methylation errors and UPD (Hattori et al., 2019; Hiura et al., 2012). One of the studies, conducted in 2015, showed that the frequency of SRS patients in an ART cohort was 8.91 times greater than

expected, however, the cohort included only 67 patients, a considerably lower number than in the non-ART cohorts. When considering the caveats associated with current data, and the poor understanding of the molecular features of SRS, it is difficult to draw definitive conclusions on the link between SRS and ART.

2.1.2 Imprinting & ART in mouse models

Many important advancements to our knowledge of genomic imprinting have been obtained from mouse models, and a particular advantage of mouse models is the high conservation of imprinting mechanisms between humans and mice (Edwards & Ferguson-Smith, 2007). The epigenetic changes, ICRs, and genes that modulate genomic imprinting are for the most part conserved in mice and humans. Mouse models were utilized to discover the consequences of faulty imprinting on the function of several genes, the modulation of critical imprinted clusters in the genome, including *Igf2/H19* and *Cdkn1c/Kcnq1ot1*, and the specific functions of many imprinted genes (Chang & Bartolomei, 2020; Cleaton et al., 2014; Edwards & Ferguson-Smith, 2007; John, 2010). Animal models have also provided useful information regarding the molecular basis of IDs. Importantly, mouse models of several ART-associated IDs have been produced, such as PWS, SRS, and AS (Davies et al., 2015; Horii et al., 2020; Lewis et al., 2019; McNamara et al., 2016).

To date, it is not entirely clear which factors of ART could affect genomic imprinting in humans, although useful information has been reported in mouse model studies. Several reports have demonstrated that superovulation led to DNA methylation aberrations at imprinted genes in embryos, oocytes, and placentas in mice (X. Chen et al., 2018; Fortier et al., 2008; Jahanbakhsh-Asl et al., 2018; Market-Velker, Zhang, et al., 2010; A. Sato et al., 2007; Velker et al., 2017; Yu et al., 2019). Notably, Chen et al., (2018) showed that superovulation had a greater effect on *Grb10* methylation and *H19* expression than in vitro maturation or IVF. Previous results from mouse models studies mirrored those obtained in human studies showing that superovulation induced DNA methylation alterations of *KCNQ1OT1, PEG1, and H19* in human oocytes (Geuns et al., 2006; Khoureiry et al., 2008; C. Sato et al., 2007). ZFP57 maintains a critical function in the control of post-fertilization mouse imprints, and it has also been reported that superovulation significantly decreased the expression of ZFP57 in mouse oocytes (Jahanbakhsh-Asl et al., 2018). The data gathered from mouse studies emphasizes that superovulation can potentially alter the expression of critical factors in oocytes that modulate post-fertilization imprints. Thus, superovulation could ultimately be conducive to the occurrence of epimutations, and consequently, IDs.

ART Procedure	Affected imprinted gene	Observed aberrations	Associated ID	Refs.
Superovulation	*Gbr10*	GOM and decreased expression of CGI1	SRS	(X. Chen et al., 2018)
	Snrpn	DMR LOM	PWS	(Saenz-de-Juano et al., 2016)
		Maternal ICR LOM		(Market-Velker, Zhang, et al., 2010)
	H19	Faulty expression	SRS	(Fauque et al., 2007)
		Expression increase		(X. Chen et al., 2018)
		Paternal allele LOM		(Market-Velker, Zhang, et al., 2010)
IVF	*H19*	Altered methylation and biallelic expression	SRS	(Tao Li, 2005)
		Maternal ICR GOM		(Tao Li, 2005)
In vitro follicle culture	*Snrpn*	DMR LOM	PWS	(Saenz-de-Juano et al., 2016)
	H19	DMR LOM	SRS	(Saenz-de-Juano et al., 2016)
	Mest	DMR LOM	SRS	(Saenz-de-Juano et al., 2016)
Ex vivo embryo culture	*H19*	Biallelic expression and LOM	SRS	(Doherty et al., 2000; Mann et al., 2004)
		ICR LOM		(S. Chen et al., 2019; Market-Velker, Fernandes, et al., 2010)
ICSI	*H19*	ICR LOM	SRS	(S. Chen et al., 2019)
	Peg3	Faulty expression and maternal DMR LOM	/	(de Waal et al., 2012)

Table 1. Expression and methylation changes of ID-associated imprinted genes after ART procedures in mouse models. Based on the table by Horánszky et al., 2021.

2.1.3 Modelling Imprinting disorders using hiPSCs

IDs and the consequences of ART procedures on the regulation of imprinted genes can also be studied using hiPSCs. There are many advantages of using hiPSCs derived from ID patients, for example, hiPSCs maintain the ID-associated genotype and furthermore, their use removes the requirement to generate genetic mutations which could induce off-target effects. The intricate and varied nature of ID aetiology means that considerable engineering is needed to comprehensively replicate their epigenetic and genetic consequences. The lineage-specific differentiation of hiPSCs derived from ID patients provides an exciting opportunity to deepen our understanding of ID effects in a range of tissues that would be challenging to retrieve from patients. By considering the information obtained from both mouse and hiPSC models, we can draw further insights into the epigenetic and genetic mechanisms underlying ART-associated IDs.

The defining characteristics of stem cells are self-renewal, proliferation, and differentiation, and in the appropriate conditions they possess the capability to differentiate into almost any cell type. Therefore, stem cells are invaluable tools for investigating early embryogenesis, disease modelling, and regenerative medicine (Ben-David et al., 2012; W. Liu et al., 2013). The reprogramming of somatic cells using ectopic reprogramming factors was a revolutionary discovery, producing patient-specific, autologous hiPSCs (Doss & Sachinidis, 2019; Rowe & Daley, 2019; K. Takahashi & Yamanaka, 2006). hiPSCs possess many features in common with human ESCs, including morphology, gene expression patterns, and epigenetic signatures, as well as the capacity for proliferation, and differentiation (Guenther et al., 2010; Mikkelsen et al., 2008; Wernig et al., 2007).

HiPSCs offer a promising platform to model diseases with complex genetic aetiologies, including IDs and their effects on brain development. The neuronal differentiation of ID patient derived hiPSCs allows for the study of the cellular and molecular disturbances inflicted by IDs on neural developmental processes. During the reprogramming procedures used to obtain hiPSCs the epigenetic landscape is reset. Since imprinted gene regulation is dependent on the proper maintenance of epigenetic marks, when modelling IDs, a comprehensive analysis of imprinting status and monoallelic gene expression is vital to confirm that the implicated epimutations are maintained in the reprogrammed ID-patient derived hiPSCs.

HiPSCs have been previously obtained from PWS and AS patients (Fink et al., 2017; Martins-Taylor et al., 2014; Yang et al., 2010). In one study, hiPSCs were derived from a PWS patient and when compared to the somatic fibroblasts of PWS patients used for reprogramming, the PWS-hiPSCs maintained similar methylation signatures at the PWS imprinting center (IC) (Martins-Taylor et al., 2014). Similarly, another study reprogrammed hiPSCs from a PWS patient and the obtained PWS-hiPSCs demonstrated the molecular characteristics of PWS including a decreased expression of imprinted genes associated with the disease and high levels of DNA methylation at the maternal PWS IC. Neuronal differentiation of PWS hiPSCs was also successfully undertaken, and therefore, they can be utilised to model the effects of PWS on the developmental processes of the embryonic brain in vitro.

The potential of hiPSCs to model IDs was also emphasised by the derivation of hiPSCs from AS patients (Fink et al., 2017). Upon neuronal differentiation, the AS hiPSC derived neuronal cultures showed no *UBE3A* expression, in-line with AS pathophysiology. Another important study using hiPSCs derived from PWS and AS patients investigated the effects of reprogramming on the chromosome 15q11-q13 (Chamberlain et al., 2010). It was reported that copy number variations at the region were unaltered by reprogramming procedures, and DNA methylation levels at the PWS IC were unaffected during reprogramming, demonstrating that methylation signatures at the PWS ICR can be maintained through reprogramming despite the significant epigenetic remodelling that occurs upon hiPSC generation. A similar study involved the derivation of hiPSCs from an AS patient with a three base pair deletion of *UBE3A* (Stanurova et al., 2016). The methylated status of the PWS-SRO DMR in the PWS IC was retained through the reprogramming process, although importantly, gains and losses of DNA methylation were reported at other DMRs, which implies a susceptibility of methylation imprints to hiPSC reprogramming and their maintenance in culture. This could have implications for our understanding of the effects of the culture and handling of gametes and embryos during ART procedures.

Currently available data suggests that ICR methylation is, for the most part, correctly recapitulated in ID patient derived hiPSCs. This implies that hiPSC reprogramming mirrors the maintenance of DNA methylation that occurs post-fertilization, rather than the epigenetic reset that occurs in the germline. HiPSCs offer an exciting platform for ID modelling, and further study can capitalise on this by determining if, from ID patients where epimutations are a causative factor, the methylation signatures of reprogrammed hiPSCs are accurately reproduced and maintained through neuronal differentiation.

To date, several studies have shown promising results relating to the use of hiPSCs to model ART-related IDs, emphasizing a broad range of investigative possibilities using ID patient derived

hiPSCs. For example, highlighting the associated molecular mechanisms and phenotype alterations. Moving forward, further investigations can focus more specifically on the dynamism of ID-linked pathological mechanisms during neuronal differentiation. For example, the neural differentiation of AS hiPSCs, to further understand the ramifications of IDs on brain development.

Presently, a significant limitation to consider when using hiPSC models is the inconsistency in the differentiation efficiency between hiPSC lines (Chamberlain et al., 2010), which could ultimately skew comparisons between studies. When considering the use of ID patient derived hiPSCs for modelling, a priority should be improving our understanding of the effects that reprogramming procedures exert on the hiPSC epigenome. Furthermore, remarkably little is known regarding the effects that reprogramming exert on imprints in patient-derived cells. Determining the stability or the susceptibility of imprints in reprogrammed cells could offer new perspectives on germline imprint properties during hiPSC reprogramming, and the active epigenetic alterations in ART-associated culture procedures linked to preimplantation development.

2.1.4 hiPSC reprogramming and methylation

As the use of hiPSCs to model IDs continues, a critical next step should be to obtain a comprehensive understanding of the effects that reprogramming exerts on the cellular epigenome. DNA methylation changes and/or LOI that occur upon hiPSC reprogramming could provide further insight into susceptibilities of various imprinted loci implicated in ART-associated IDs. The global DNA demethylation that occurs during mammalian development in the germ line and embryo also occurs during hiPSC reprogramming in vitro (Hill et al., 2014). It has been previously reported that reprogramming can induce genetic and epigenetic aberrations in hiPSCs, while imprints are particularly vulnerable to reprogramming procedures (Bar et al., 2017; Godini et al., 2018; Johannesson et al., 2014; X. Li et al., 2019; Perrera & Martello, 2019).

It has been previously shown that somatic cell reprogramming via the over-expression of reprogramming factors (*Sox2, c-Myc, Oct3/4, Klf4)* has produced hiPSCs that preserve an epigenetic memory of the somatic cell methylation status, and that also show different methylation signatures to ESCs (Ma et al., 2014). Additionally, it was observed that the reprogramming efficiency determines the extent of methylation alterations in hiPSCs in comparison to the somatic cells of origin, and there are continued attempts to improve reprogramming efficiencies (Ruiz et al., 2012; Wen et al., 2016).

HiPSC-specific DMRs are acquired during reprogramming (Bar-Nur et al., 2011; Lister et al., 2011) and are mainly linked with CpG islands and genes. It is currently postulated that these DMRs are partly comprised of hiPSC-specific methylation marks as well as an epigenetic memory of the original somatic cell. Intriguingly, hiPSC-specific DMRs have been identified in independent hiPSC lines, which implies that the certain loci may be particularly susceptible to methylation changes upon hiPSC reprogramming. Since genomic imprinting fundamentally relies on the establishment of DMRs at specified loci in gametes (Bar & Benvenisty, 2019), further study of the vulnerabilities of DMRs to altered methylation in hiPSCs can provide an enhanced understanding of how ART procedures induce LOI by comparing patterns of susceptibilities between LOI observed in ART patients and hiPSCs.

The factors to consider when observing the variability of DMRs between hiPSC lines include culture conditions (Lister et al., 2011; Vaskova et al., 2013), donor somatic cell age and genetic background (Jones et al., 2015; Rouhani et al., 2014; Sun & Yi, 2015), the method used to derive the hiPSCs (Ma et al., 2014), and the number of passages (Shan et al., 2014). Moreover, acknowledging the role of imprinted genes in the dosage control of cell proliferation it is also likely that selection contributes to the status of DMRs in culture. Earlier reports observed that, intriguingly, prolonged periods in culture curtailed the differences in methylation signatures between hESCs and hiPSCs, although epigenetic defects can also occur after prolonged culture (Nishino & Umezawa, 2016; Tesarova et al., 2016).

2.1.5 hiPSC imprinting status.

Human pluripotent stem cells obtained using reprogramming methods, such as hiPSCs and nuclear transfer embryonic stem cells (ntESCs) have been reported to be more susceptible to LOI when compared to hESCs. Furthermore, evidence suggests that certain imprinted loci display an increased vulnerability to LOI (Bar et al., 2017; Johannesson et al., 2014; Ma et al., 2014; Pick et al., 2009; Takikawa et al., 2013). There have been conflicting reports on the effects of reprogramming on iPSC imprints. For example, it has been shown that LOI is not a common occurrence in hiPSCs (Hiura et al., 2013). In contrast, the *Dlk1/Dio3* imprinted was hypermethylated in mouse iPSCs resulting in aberrant gene expression (Stadtfeld et al., 2010), which suggests that the reprogramming procedure can affect imprints in reprogrammed cells.

DNA methylation signatures at imprinted loci in hiPSCs are potentially less vulnerable to aberrations from reprogramming and in culture than methylation marks at non-imprinted areas of

the genome (Rulands et al., 2018; Shipony et al., 2014; Takikawa et al., 2013; Yagi et al., 2019). This suggests that these imprinted loci are distinctively regulated in a similar manner to imprints in vivo during the PC period allowing for the preservation of the parental methylation signatures.

During extended periods of culture, hiPSC imprinting status is maintained, regardless of whether LOI has occurred (Bar et al., 2017; Hiura et al., 2013). Importantly, as occurs during in vivo development, faulty imprints have been observed to be retained during lineage specific differentiation of hiPSCs. An important consideration when investigating the use of hiPSCs for reliable disease modelling is the observation that their imprinting status is maintained during lineage-specific differentiation, and LOI could occur in reprogrammed hiPSCs.

Despite contradictory reports, many studies have shown that hiPSC reprogramming could alter imprinted DMRs (Brix et al., 2015). Given that many imprinted genes are situated in clusters that are regulated by a common DMR, an error to a DMR leading to a LOI could consequently affect the gene expression/biallelic expression of several genes within the cluster (Bartolomei & Ferguson-Smith, 2011; Ishida & Moore, 2013). The LOI that can occur during hiPSC reprogramming is thought to be partly due to the action of Ten-eleven translocation methylcytosine dioxygenase (TET) proteins that catalyse 5-methylcytosine (5-mC) oxidation to 5-hydroxymethylcytosine (5-hmC). During hiPSC derivation, 5-hmC levels significantly increase, which also occurs during in vivo epigenetic reprogramming. This increase can likely be attributed to increased expression of the proteins TET1 and TET2 as previous observations have shown that deficiencies of these proteins results in a decrease to hiPSC reprogramming efficiency (Hu et al., 2014). LOI at the H19 imprinted locus during hiPSC reprogramming was previously postulated to be a mediated by TET proteins, although there are potentially several complex factors involved in modulating methylation signatures during reprogramming procedures (Bermejo-Álvarez et al., 2015; Hill et al., 2014).

The biallelic expression of imprinted genes has been demonstrated to be altered in many human pluripotent stem cell types. For example, *H19, PEG3, MEG3, MEST, IGF2,* and *PEG10* have frequently exhibited biallelic expression in various hiPSC lines (Johannesson et al., 2014; Ma et al., 2014; Nazor et al., 2012; Pick et al., 2009). Bar and colleagues (2017) performed a large-scale study of LOI in several hiPSC lines and observed that the occurrence of LOI was most frequent in the *MEG3/DLK1*, *Zdbf2/GPR*, and *H19/IFG2:IG-DMR* imprinted regions. Intriguingly, these imprinted loci are commonly regulated by a paternally methylated DMR, though importantly, it is likely that the methylation mark at *Zdbf2* is a somatic DMR and is as such unlikely to be altered by ART procedures (Duffie et al., 2014). It is currently understood that 23 DMRs are established in the germline, with 3 of those present on the paternally inherited chromosome and the remainder

being methylated in the maternal germline (Kelsey & Feil, 2013). Evidence from previous studies implies that imprinted loci under the control of paternally methylated DMRs have an elevated susceptibility to LOI than those regulated via maternally methylated DMRs (Bar et al., 2017; Rugg-Gunn et al., 2007). Moreover, the increased vulnerability of imprinted genes regulated by paternally methylated DMRs in hiPSCs suggests that paternally methylated regions may be more sensitive to aberrations due to reprogramming procedures.

A potential explanation for vulnerabilities of certain imprinted genes involves their ability to withstand the erasure of methylation marks during the pre-implantation developmental stages (Rugg-Gunn et al., 2007). The preservation of parental epigenetic memory relies on the maintenance of methylation signatures at DMRs during early development; Zinc finger proteins 445 (ZNF445) and Zinc finger protein 57 homolog (ZFP57) are both key modifiers with critical functions in imprint maintenance during the genome-wide epigenetic reset that occurs post-fertilization.

It has been shown that zygotic ZFP57 is vital for DNA methylation maintenance at certain imprinted genes during hiPSC reprogramming, for example, *SNRPN* and *DLK1/DIO3* (McDonald et al., 2016). However, ZFP57 was not needed at other imprinted regions for methylation maintenance, such as *PEG1 and PEG3*, which are postulated to be under the regulation of ZNF445 (N. Takahashi et al., 2019). Altered expression of these zinc finger proteins, therefore, could lead to methylation errors at imprinted loci in pluripotent stem cells. ZFP57 expression in mice is associated with the pluripotent state, as higher expression levels are observed in oocytes and the early embryo, followed by a gradual decline in expression as lineage-specific growth and differentiation occurs. In somatic cells, ZFP57 expression ceases (X. Li et al., 2008; Loh et al., 2007). This provides a potential explanation for the heightened vulnerability of hiPSCs to imprinting errors compared to ESCs; somatic cells are used for hiPSC derivation, which have decreased protective capabilities over imprinted loci. Aberrant regulation of TET proteins and DNA methyltransferases (DNMTs) in culture remains a distinct possibility, which would result in changes to ZFP binding sites, leading to demethylation of the ZFP57 binding motif that would ultimately render it unrecognizable by ZFP57. As a result, DNMTs and Krab-associated protein 1 (KAP1) would not be recruited to maintain DNA methylation (Voon & Gibbons, 2016).

In addition to explaining imprint aberrations in hiPSCs, irregular zinc finger protein expression could also play a role in the imprinting errors described in ART patients leading to an increased incidence of IDs. Further study into the consequences that ART procedures exert on ZNF445 and ZFP57 regulation could reveal critical information on the elevated levels of IDs among ART patients. Furthermore, an interesting direction for future investigations could be to measure the

regulation of zinc finger proteins in hiPSC cultures and assess whether they contribute to the LOI observed in hiPSCs.

Reports of changes to imprinted regions in hiPSCs are accompanied by studies into the methylation profiles of DMRs that regulate imprinting genes in ART populations. It has been previously observed that ART-conceived (IVF/ICSI) children showed increased methylation at *PEG3* DMRs and decreased methylation levels at *H19/IGF2:IG* DMRs (Barberet et al., 2021). Additional results from the same study also suggested that the methylation status of *MEG3* DMRs are sensitive to ART procedures. Interestingly, methylation alterations at *PEG3* and *H19/IGF2:IG* DMRs were also observed in mouse models of ART (Table 1).

To date the majority of studies using ID patient-derived hiPSCs have reported that the methylated status is faithfully recapitulated in derived hiPSCs compared to the somatic source. Promisingly, this means that patient-derived hiPSCs could be useful for modelling epimutation-associated IDs.

2.2 hiPSC models and NCDs of the brain

The development of iPSC technology, introduced by Takahashi et al. 2007, allowed for disease modelling, drug screening, and therapy development on patient-specific cell lines (Shi et al., 2017; K. Takahashi & Yamanaka, 2006), whilst also providing a powerful tool in the field of regenerative medicine (Hirschi et al., 2014). The hiPSCs generated from this process share pluripotency and self-renewal attributes identical to those of embryonic stem cells. Research has capitalised on patient-specific hiPSCs to further the understanding of the mechanisms and pathophysiology of diseases, while they also have the potential to provide a limitless source of cells for 'disease-in-a-dish' models. Furthermore, hiPSCs are an invaluable resource of neuronal cells (Shi et al., 2017), that were previously difficult, or impossible to obtain. Due to advancements in genetic manipulation and in vitro differentiation techniques, hiPSCs are becoming increasingly useful for improving the understanding of human embryonic development, which can assist in the prediction and/or prevention of developmental deficiencies that arise due to environmental or genetic factors (Zhu & Huangfu, 2013). A beneficial advantage of hiPSCs reprogrammed from somatic cells is their retention of the genomic features (gene mutations, chromosomal abnormalities) of the patient (L. Li et al., 2018). A thorough analysis of imprinted genes should be undertaken on hiPSC lines, as many published hiPSCs that pass the required reprogramming criteria have demonstrated imprinted gene expression errors as well as aberrant DMR methylation patterns. This is

particularly relevant for disease modelling because aberrant imprinting can lead to confused conclusions with regards to disease phenotyping (Hiura et al., 2013).

Non-communicable diseases (NCDs) are responsible for the highest number of deaths worldwide. The majority of NCDs are induced by preventable factors, such as high alcohol consumption, poor diet, and tobacco use. NCDs were responsible for 41 million deaths globally in 2023 alone (World Health Organization, 2023), and the prevalence of neurological disorders seems to be increasing. Although the direct reason for the increase is unknown, environmental factors are believed to contribute (Sirenko et al., 2019), as well as genetic defects, and chemical exposures (Grandjean & Landrigan, 2006). There is a pressing need to successfully identify the determining factors of NCDs during the human lifetime. The periconceptional period is particularly important, as cumulative supporting evidence of the DOHaD hypothesis indicates that interventions to early-life environments provide a crucial opportunity for the prevention of DOHaD-linked NCDs (Bay et al., 2019).

As the average age of the human population increases, so too does the prevalence of neurological disorders. Early-life stress affecting brain development can result in alterations to its architecture; schizophrenia, autism and even Alzheimer's and Huntington's disease are considered as neurodevelopmental disorders. Millions of people are affected by these disorders annually, yet there is currently no therapeutic cure (Centeno et al., 2018). The exact mechanisms underlying the onset of such neurodevelopmental disorders are still under investigation. Much evidence suggests that insults during fetal neurodevelopment, such as maternal stress (and the associated endocrine fluctuations), nutrient deficiency, and infection inflict long-term changes on the concurrent neural development of an organism that can result in neurological and behavioural alterations (Van Den Bergh, 2011).

iPSC-derived neuronal cellular models are critical to increasing knowledge of the mechanisms of neurological disease and provide the potential for developing therapies to treat such diseases in a patient specific manner. There are many available differentiation protocols for hiPSC-derived neuronal cultures, and current methods allow the production of all types of neural cells with relevant functional and physiological characteristics that can be used for disease modelling The generated neuronal cultures are self-renewable and can be differentiated into many glial/neuronal subtypes, whilst they can also be used to mimic the developmental processes of the brain (Bal-Price et al., 2018).

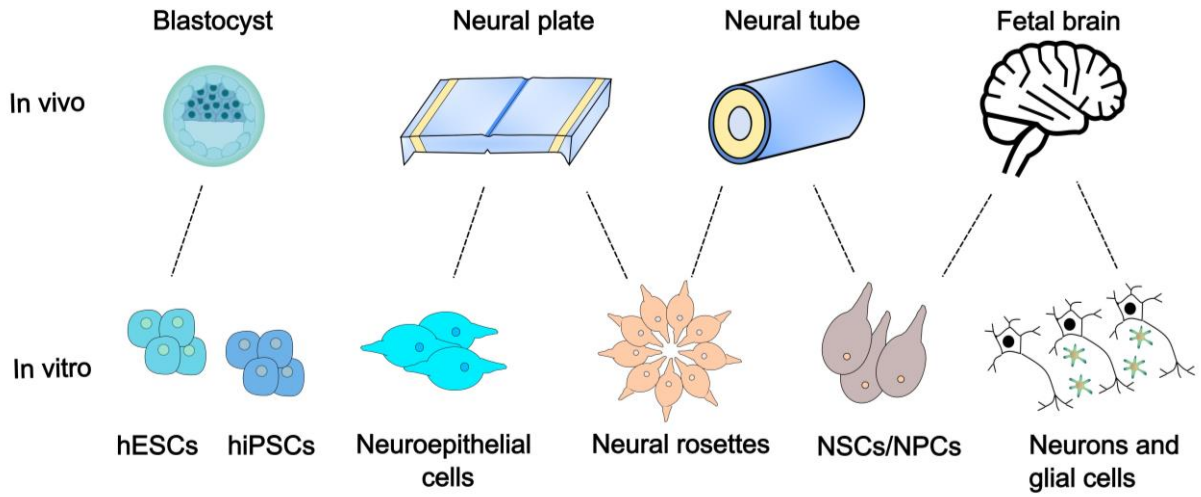

Figure 3. Overview of the initial stages of brain development from blastocyst to fetal brain as well as the representative in vitro stages. (Images of neurons, blastocyst and brain obtained from Wikimedia, image of astrocyte obtained from Reactome (Bio). All images used under creative commons licenses).

HiPSC-derived neuronal cultures allow for the recapitulation of in vivo conditions, producing physiologically representative models of the CNS in vitro that are invaluable for neurological disease-related investigations (Centeno et al., 2018). 2D cell culture has provided valuable, simple, and low-cost platforms for the modelling of such diseases for over a century (Blain et al., 2009). It is argued, however, that 2D models cannot fully replicate the architectural intricacies of the brain, paving the way for the application of 3D models. It is postulated that 3D models allow for a closer representation of in vivo CNS architecture, and therefore, are an appropriate option to link animal models and 2D cultures. There is already evidence supporting the superiority of 3D cultures over 2D in several aspects, including cell-cell connections, cell differentiation and electrophysiological network characteristics (Antoni et al., 2015; Hopkins et al., 2015). Previously, studies involving 3D neuronal differentiation have generated organoids (Lancaster & Knoblich, 2014) or fixed NPCs into scaffold-based systems (Y. Zhang et al., 2016).

A favourable protocol for the neural induction of hiPSCs utilises Noggin and SB431542, inhibitors of Mothers against decapentaplegic (SMAD) signalling, which result in a rapid neural conversion. The dual-SMAD inhibition technique allows for the use of hiPSCs in disease modelling without the need for embryoid bodies and stromal feeders which both have associated disadvantages (Chambers et al., 2009). A characteristic feature in the production of NPCs is the presence of neural rosettes; morphologically distinctive structures that contain NPCs. The development of neural rosettes is reflective of neural tube formation, or neurulation during embryonic CNS development (Fedorova et al., 2019). The differentiation and maintenance of neural stem cells (NSCs) and neural progenitor cells in the developing human CNS is instructed via cues that lead

them to differentiate into specialised neuronal and glial cells (Maldonado-Soto et al., 2014; Preston, 2011). There remain, however, challenges in producing specific neuronal subtypes from the differentiation process, as there is a high degree of variation in produced cultures. There are many factors that potentially contribute to the observed variations, such as differences in culture media, different handling, and heterogeneity in the original cells (Engler et al., 2006). As methods to differentiate hiPSCs into neuronal cultures continue to improve, they increase the prospect of accurate knowledge extraction from neurodevelopmental studies that utilise them.

2.3 Environmental chemical exposures during periconceptional period: modelling effects on brain development

The definition of neurotoxicity, in general terms, is any negative effect on the central (CNS), or peripheral nervous systems (PNS), which can be induced by biological, chemical, or physical means (Costa, 2008). Developmental neurotoxicity (DNT) specifies the effects of xenobiotic exposure on the nervous system during its development (Coecke et al., 2007). Several methods, whether *in vitro/vivo,* have been utilised to investigate the toxicological mechanisms (e.g., altered gene expression, cytotoxicity, mutagenesis) that induce the pathogenesis of many diseases (Kang & Trosko, 2011). DNT studies investigating the effects of environmental chemicals on the developmental processes of the brain fall firmly within the DOHaD bracket. These investigations are useful for predicting the long-term effects of environmental chemical exposures on the development of the brain. Furthermore, any developmental clues/links to NDDs and NDs identified in DNT studies consolidate the DOHaD hypothesis and offer future promise for efficient disease prediction.

It is of vital importance that a reliable and accurate model for DNT and neurotoxicity evaluation contains the specific cell types of the CNS at the appropriate developmental stage, and that any model used for testing truly represents its complex nature. The specific properties of the CNS and brain leads to differences when studying neurotoxicity compared to toxicity in other organs (hepatotoxicity, cardiotoxicity etc.), for example, the presence of the blood brain barrier (BBB), a high proportion of functionally important lipids (Veloso et al., 2011), and exceptionally high energy demands; great amounts of ATP are required for the maintenance and generation of neuronal membrane potentials.

The developing nervous system is extremely vulnerable to neurotoxic compounds and mutagenic insults compared to adult brain networks. During its development, the nervous system undergoes

a highly complicated and temporally co-ordinated process. The complexity of the nervous system, along with an extremely high level of gene expression during development renders it more susceptible to damage from chemical exposures than the fully matured nervous system of an adult (Grandjean & Landrigan, 2006). Because toxicity in the developing brain is fundamentally different from adult neurotoxicity, DNT testing requires substantially different cellular sources, experimental controls, and endpoints from NT screening batteries. Drugs (Zhong et al., 2020), pesticides (Pamies et al., 2018) and industrial chemicals (Li et al., 2019) not only disrupt normal brain development, but trigger molecular initiating events (MIEs) (mitochondrial dysfunction, protein aggregation, genetic lesions, cell death) causing neurodegenerative disorders, such as Parkinson's disease (PD) (Lucchini et al., 2017), Alzheimer's disease (AD) (Caito & Aschner, 2017) and epilepsy (Prox et al., 2013). Whether DNT manifests due to chemical exposure or genetic lesions, the adverse outcome exacerbates in neural circuit development by disruption of key neurodevelopmental processes. Given that such developmental mechanisms are effectively recapitulated in vitro, these spatiotemporally distinct processes can serve as key events for DNT assays (Fritsche et al., 2018). Thus, neuroepithelial precursor development, NPC proliferation, neural rosette formation, apoptosis, neural crest cell migration, neural/ glial differentiation, neurite growth, synaptogenesis and network activity are utilised as most common functional endpoints of a DNT screening. Therefore, analytical endpoints might be analyses of neural and glial marker expression, high content image analyses of morphological features, cell viability, proliferation, and apoptosis assays.

To date, neurotoxicity and DNT studies have been conducted using mainly in *vivo* models. Conducting research using animal models can lead to high costs and requires animal sacrifice. Using results from in *vivo* studies using animal models to predict human outcomes is also limited by species disparity; toxicity data extrapolated from mouse models may differ completely in humans (Szebényi et al., 2011). Developing representative and reliable in *vitro* human testing models for DNT and neurotoxicity is currently an essential avenue for research, as it will allow for the efficient screening of the toxicological effects on neuronal development, and disease outcomes in the nervous system. Researchers, however, have a limited access to human tissue, and it is even more difficult to acquire tissues that have not been previously exposed to drugs, disease or other factors. Because of this, researchers have focused on the use of neural cultures derived from hiPSCs for neurotoxicity and DNT investigations (Bosnjak, 2012).

HiPSC technology can be utilised as a robust investigatory tool in the DNT field. NSCs are used to produce human in vitro neuronal cultures, which are being used extensively in modern research as they can differentiate into many glial and neuronal subtypes (Breier et al., 2010), and they have

self-renewing abilities. HiPSCs can be used to acquire NPCs, and due to the ethical complications and differing legislations that regulate ESC use, as well as their broad potential for in vitro toxicological applications, glial and neuronal testing models derived from hiPSCs are becoming ever more popular for use in research. HiPSCs can proliferate in culture before differentiating into a diverse range of cell types, which includes NSCs, neurons and glia; this allows biological mechanisms that are specific to a tissue to be quantified in vitro via high-throughput methods (Scott et al., 2013). High-throughput methods are critical for knowledge advancement in the field of predictive neurotoxicity.

2.3.1 NSCs in the developing brain

In vertebrates, CNS development begins when the neural plate folds, forming the neural tube which is initially comprised of a single layer of neuroepithelial cells. Upon maturation of the neural tube, stratification occurs, altering the tissue architecture. NSCs are then located on the ventricular layer, and the migration of post-mitotic cells towards the surface of the brain occurs (Altmann & Brivanlou, 2001). This organisational process, typically described in relation to cerebral cortex development, is maintained throughout embryogenesis; in the germinal zones of the ventricles proliferating cells are located, while mature progeny migrate to their final destinations. This process also occurs in other regions of the brain.

The first stage of fetal CNS development, known as primary neurulation, is when NSCs first arise in the developing brain and the neuroectoderm develops from the ectodermal germ layer (Leibovitz et al., 2022; Nikolopoulou et al., 2017). The differentiation of multipotent, self-renewing NSCs is a strictly controlled and temporally coordinated process that ultimately gives rise to the neuronal and glial cell types of the human brain (Obernier & Alvarez-Buylla, 2019; Temple, 2001). In the early phases of neurogenesis, NSC expansion via symmetric and asymmetric cell divisions initially promotes brain development, resulting in an NSC and an intermediate neural progenitor (NPC). As they enter a "primed" state for neuronal differentiation, NPCs have a lower proliferative capacity than NSCs, but their division is maintained throughout neurogenesis, creating a replenishing pool of progenitors for further neuronal/glial specification (Bergström & Forsberg-Nilsson, 2012; Suzuki et al., 2021).

NSCs undergo dynamic alterations to their morphology and characteristics during development. For instance, upon the initiation of neurogenesis, radial glial cells gradually replace neuroepithelial cells (Malatesta et al., 2000). Radial glia possess soma that lie within the periventricular zone, and

processes that extend to the pial surface from the ventricle. Radial glia typically exhibit many properties that overlap with astroglia, and they express characteristic markers of glial lineage, like radial-glial cell marker-2 (RC2) (Misson et al., 1988). In addition to providing neuronal and glial cells via asymmetric divisions, radial glia also facilitate neuronal migration as an acting scaffold (Bergström & Forsberg-Nilsson, 2012). It is currently accepted that radial glial cells are classified as NSCs, and they share many characteristics with neuroepithelial cells. During the cell cycle, radial glia show interkinetic nuclear migration while they also have a polarised organisation. Moreover, it has been shown that, in comparison to neuroepithelial cells, radial glia have a more restricted differentiative potential. Retroviral trace tagging in vivo has previously been used to show the tri-potent property of neuroepithelial cells, while in contrast, radial glial cells differentiate into single cell types only (e.g., neurons, oligodendrocytes, astrocytes) (Grove et al., 1993; McCarthy et al., 2001; Reid et al., 1995). Although it was initially difficult to accept radial glial cells as a type of NSC, gradually increasing evidence supported this conclusion. Embryonic, postnatal, and adult NSCs share many characteristics with astrocytes. Importantly, only a small percentage of astrocytes in the postnatal brain have the same functionality as stem cells (Bergström & Forsberg-Nilsson, 2012).

2.3.2 Bisphenol A

BPA is commonly utilised as a chemical precursor in the industrial production of polycarbonate plastics, food and drink packaging, and thermal paper, which are commonly found in daily life (Porras et al., 2014; Vandenberg et al., 2007). As an endocrine disrupting compound, BPA has the capacity to imitate, obstruct, or modify the activity of endogenous hormones. Relevant to the DOHaD theory, BPA can cross placenta and reach the foetus, while newborns can be exposed to BPA via breast milk. Additionally, BPA has been found in human urine, blood, amniotic fluid, fetal serum, and breast milk. Upon human ingestion via the gastrointestinal (GI) tract, BPA is metabolised to BPA glucorinide and BPA sulfate (Mao et al., 2023; Rebolledo-Solleiro et al., 2021).

According to estimates, BPA consumption in humans ranges from 1 to 5 µg/kg/day (Murata & Kang, 2018), and many individuals are exposed to it on a chronic basis due to its environmental prevalence (Welch & Mulligan, 2022). Importantly, earlier research shows that BPA causes abnormalities in brain development in animal models at levels in the ng/kg/day range, which is substantially lower than the predicted daily consumption for humans (Negri-Cesi, 2015; Welch & Mulligan, 2022).

2.3.3 BPA: Effects on brain development

Previous studies suggest that BPA exposure is associated with NDDs, such as ASD, NDs including PD and AD, as well as impaired cognitive function and behaviour (Robolleido-Solleiro D et al., 2020). It has also been reported that BPA exposure is linked to abnormalities in the structure and function of the CNS, arising because of its endocrine disrupting properties that enable it to affect various molecular pathways that are critical for brain development. Due to its lipophilic properties, BPA can permeate cell membranes, the placenta, and the fetal BBB, allowing it to disrupt the embryonic and fetal stages of CNS development (Balakrishnan et al., 2010; Ikezuki et al., 2002). BPA's ability to permeate cell membranes, the placenta, and the fetal blood-brain barrier (BBB) allows it to disrupt the development of the embryonic and fetal nervous systems (O'Shaughnessyhich et al., 2021). Notably, a human fetal investigation of free/conjugated BPA revealed a decreased fetal capacity to metabolise BPA (Nahar et al., 2013), which may suggest that the developing foetus is more susceptible to the negative effects of exposure.

The brain is reportedly a common target of endocrine disrupting compounds (Gore, 2010), which is in accordance with the fact that early-life BPA exposure is linked with NDDs and behavioural abnormalities in children (de Cock et al., 2012; Minatoya & Kishi, 2021; Mustieles & Fernández, 2020). There is a positive association between maternal BPA exposure levels during pregnancy and symptoms in children such as externalizing behaviours (Stacy et al., 2017), learning difficulties (Jensen et al., 2019; Lin et al., 2017), attention deficit hyperactivity disorder (ADHD) (Jensen et al., 2019), anxiety and depression (Casas et al., 2015). Currently, there remains a lack of data into the effects of BPA on the structure and function of the human brain. However, several investigations have been carried out into the neurotoxic effects of BPA on animals in vivo and in vitro, shedding light on the cellular and molecular alterations induced by BPA during brain developmental processes that could contribute to NDDs.

Synaptogenesis occurs when a neuron responds to guiding signals by extending its axon towards a target cell, typically a muscle cell, gland, or neuron, forming an adhesion that facilitates neuronal communication via synaptic connection. The specialised neural networks of the PNS and CNS are developed via synaptogenesis, therefore, proper synaptic development is a prerequisite for appropriate behavioural response processes in the brain (Batool et al., 2019; Poggio, 1990; Taverna et al., 2014).

Aberrations to synaptogenesis can lead to NDDs, therefore, clarifying the effects of BPA exposure on synapse formation is vital as BPA-induced synaptic impairments could lead to the onset of NDDs (Batool et al., 2019). Axonal guidance and growth has previously been demonstrated to be

altered by BPA in zebrafish and fruit fly models. Similarly, BPA treatment of hESC-derived NSCs resulted in decreased neurite growth (Liang et al., 2020; Morrice et al., 2018; X. Wang et al., 2013). On the other hand, examination of dendritogenesis in rat hippocampus neurons in vitro revealed that BPA administration enhanced the motility and density of dendritic filipodia as well as dendrite length. BPA can impact synaptogenesis once the presynaptic and postsynaptic membranes come into contact, according to studies on synaptic morphology. After neonatal BPA exposure, analysis of the hippocampus in male mice revealed changed synaptic structural properties, for example, increased size of synaptic clefts, decreased active zones, and increases to synaptic curvature (Xu et al., 2013, 2014).

BPA treatment between 50µM and 200µM has previously been demonstrated to induce decreased synapse number and cell size of mouse neuroblasts in vitro (Yin et al., 2020). Additionally, BPA exposure reportedly caused decreases to Drebin (Dbn), Tubulin associated unit (Tau) and Microtubule associated protein 2 (MAP2) expression, as well as an increase to Synaptophysin (SYP) expression in mouse neuronal cells in culture (Van Battum et al., 2015). The relevance of these BPA-induced alterations is highlighted when considering the molecular functions of these proteins. Both SYP and Dbn modulate the morphological characteristics of synapses, while Tau and MAP2 are important for stabilizing the neuronal cytoskeleton. Therefore, the changes to the expression of these proteins invoked via neuronal exposure to BPA adds clarification to the damaging molecular consequences that this compound exerts on synaptic development and maintenance. As well as in vitro evidence, in vivo studies using both vertebrate and invertebrate models have contributed evidence that emphasises BPA's capacity to induce aberrations in synaptic development. For example, it has been shown that BPA can cause erroneous formation of axons, dendrites, and aberrations to synaptic structure, while the expression of genes crucial for synaptic development has been shown to be altered in mammalian and invertebrate model systems (Liang et al., 2020; Van Battum et al., 2015). This implies that synaptic impairments due to BPA exposure could at least partially be explained by aberrations to vital genes in the neurodevelopmental program.

2.3.4 BPA effects on NSCs

Many studies have investigated the effects of BPA exposure on NSC populations to further the understanding of its impacts on the prenatal stages of brain development. According to studies conducted in vitro and in vivo, NSC proliferation can increase or decrease depending on the dose and length of exposure to BPA. Notably, the majority of in vitro investigations employed BPA

treatments lasting between 24 hours and 7 days to simulate the effects of acute exposure on already established NSC populations (Agarwal et al., 2016; Dong et al., 2021; Gill & Kumara, 2021; Huang et al., 2019; K. Kim et al., 2007; Tiwari et al., 2015). It is crucial to assess BPA's acute toxicity to safeguard the environment and the general public's health. However, more accurate in vitro models that can predict BPA's long-term effects are required for a better risk assessment of the substance's effects on brain development and possible contributions to NDs and NDDs. Importantly, cellular responses to BPA may be significantly more complex in chronic exposure situations with sub-cytotoxic concentrations. This is especially true given that BPA is known to exert its intracellular effects by interfering with a variety of receptors, including noncanonical steroid hormone receptors and nuclear receptors (Acconcia et al., 2015).

Neurogenesis and optimal brain functions depend on a closely controlled balance between NSC proliferation and differentiation, and NDDs typically show alterations to this equilibrium (Casas Gimeno & Paridaen, 2022; E. S. Chen et al., 2014; Ernst, 2016). A number of studies have already shown that BPA disrupts these processes by interfering with crucial signalling pathways in NSC development, including the Wnt/B-Catenin, estrogen-related receptor (ERR), transforming growth factor beta (TGF-β), c-Jun N-terminal kinase (JNK), cAMP response-element binding (CREB), and tumour protein 53 (p53) pathways (Dong et al., 2021; R. Liu et al., 2013; Tiwari et al., 2015, 2016; Welch & Mulligan, 2022).

Despite widespread interest and significant research, the processes underpinning BPA-induced changes to NSCs and their potential links to abnormal brain development, NDDs and NDs, remain controversial and need to be clarified. Modern technologies are used in quantitative proteomic techniques to examine cellular proteome changes following exposure to environmental chemicals, clarifying toxicity processes, and enhancing chemical risk assessments (Gao et al., 2009). Methods based on proteomics have an advantage over those based on transcriptomics since they directly measure protein levels rather than transcript levels. Therefore, a better knowledge of the impact of BPA exposure on the expression levels of proteins of interest can be obtained using proteomic methods.

By bridging the knowledge gap between data from animal models and human research, the neuronal differentiation of hiPSCs offers an invaluable platform for future investigation of the impact of environmental chemicals on the developmental processes of the human CNS (J. Xie et al., 2020). Compared to 2D cell culture systems, 3D cultures more closely replicate complex human tissues of interest in terms of cell signalling, differentiation capacity, and tissue organisation. They also show more realistic responses to environmental chemicals (Kobolak et al., 2020; H. Wang et al., 2023). The crucial neurulation stage of embryonic brain development, where

NSCs are first produced in the developing brain, may be accurately modeled in vitro via the neural induction of hiPSCs to NSCs (Chambers et al., 2009; Galiakberova & Dashinimaev, 2020).

2.3.5 BPA: Mechanisms of action

It is widely accepted that exposure to BPA can consequentially interfere with the function of estrogen and androgen receptors (Welshons et al., 2006). Numerous endocrine pathways are affected by these changes (Rebolledo-Solleiro, 2021), which could also potentially affect the neuroendocrine processes of the brain and contribute to both NDs and NDDs. BPA binds to ERs, inducing either agonistic or antagonistic effects depending on a range of factors including the cell type, tissue and specific subtype of ER (Welshons et al., 2006). Additionally, BPA is a known antagonist of androgen receptors (ARs) (Wetherill et al., 2007). BPA prevents the nuclear translocation of ARs, also impacting their functions by several mechanisms (Teng et al., 2013). Aryl hydrocarbon receptors (Arh) are transcription factors involved with the regulation of the effects induced by polyaromatic hydrocarbons. Arh's modulate xenobiotic metabolism, and BPA has been demonstrated to decrease their activity, while interestingly, following gestational BPA exposure, there is an increased transcript expression of Arh the brain (Bonefeld-Jørgensen et al., 2007; NISHIZAWA et al., 2005).

BPA has also been reported to interact with thyroid hormone receptors (TRs), inhibiting their transcriptional actions as well as their expression levels in both in vivo and in vitro settings (Gentilcore et al., 2013; Sheng et al., 2012). It has also been discovered that BPA alters the hypothalamic-pituitary-adrenal (HPA) axis, in a sex-dependent manner, and can affect glucocorticoid receptors (GR). It was reported females treated with BPA exhibited an increase to their corticosterone levels when compared to men treated with BPA. Moreover, both female and male rats demonstrate higher corticosterone levels upon stress after BPA exposure, while female GR levels were increased compared to males (Panagiotidou et al., 2014; Poimenova et al., 2010).

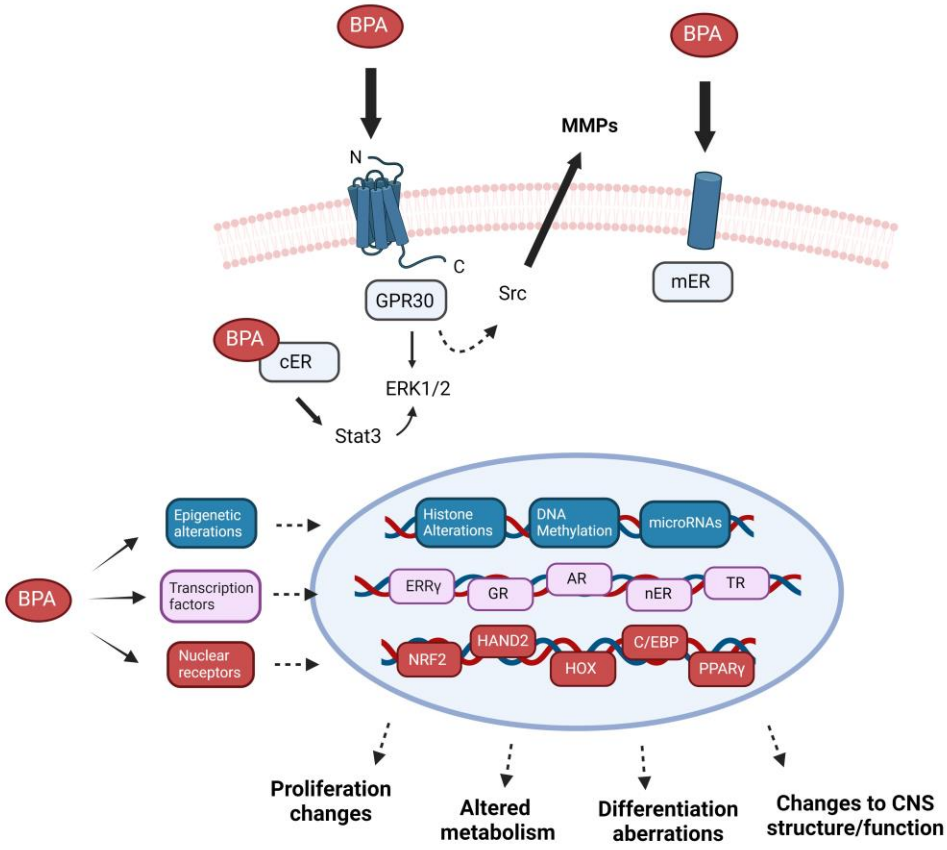

Figure 4. A model for the molecular actions and phenotypic alterations induced by BPA exposure. BPA has the capacity to cause changes to cellular proliferation, differentiation, and metabolism, while it has also the ability to cause alterations to the structure and function of the CNS. These effects are induced when BPA activates specific receptors, such as transcription factors, while epigenetic changes can also be observed after BPA exposure (Cimmino et al., 2020).

Importantly, the mechanisms discussed thus far play roles in the regulation of specific patterns of behaviour, for example, ERs and ARs are associated with sexual behaviour, while ERs and GRs are linked to learning and memory, as well as anxiety and depression. Changes to the expression levels of the molecules in question would, in theory, also be accompanied by behavioural alterations (Rebolledo-Solleiro, 2021). The expression of the discussed molecules is specifically regulated based on age, tissue, specific region of the brain, or sex endocrine condition, thus rendering a complex picture to understand the effects of BPA's effects on the brain during embryonic development. Furthermore, the discussed molecules can exert a broad variety of downstream physiological effects depending on which specific receptors are activated.

3 MATERIALS AND METHODS

3.1 Chemicals and plasticware

Sigma-Aldrich (St Louis, MO, USA) was used to obtain all chemicals. Except when otherwise noted, all plasticware and cell culture supplies were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

3.2 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 Cat#: 85850) and BD Matrigel™ matrix (BD Biosciences, Cat#: 356234) 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, Cat#: 11-7024) 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).

3.3 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 (Cat#: 17502048), 2x B27 (Cat#: 12587010), 100µM β-mercaptoethanol (Cat#: 31350010), 2mM L-glutamine (Cat#: G7513), 1x non-essential amino acid (NEAA, Cat#: M7145), 5µg/mL insulin (Cat#: I9278) supplemented with 200nM LDN-193189 HCL (Selleck Chemicals, cat# S7507), 10µM SB431542 (Cat#: S4317), and 5ng/mL basic fibroblast growth factor (bFGF, Cat#: PHG0261). On Prime Surface 96well V plates (PHC Europe, Cat#: MS9096VZ) in 200µL NIM

the following day, hiPSCs were dissociated with Accutase® (Cat#: A6964) 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.

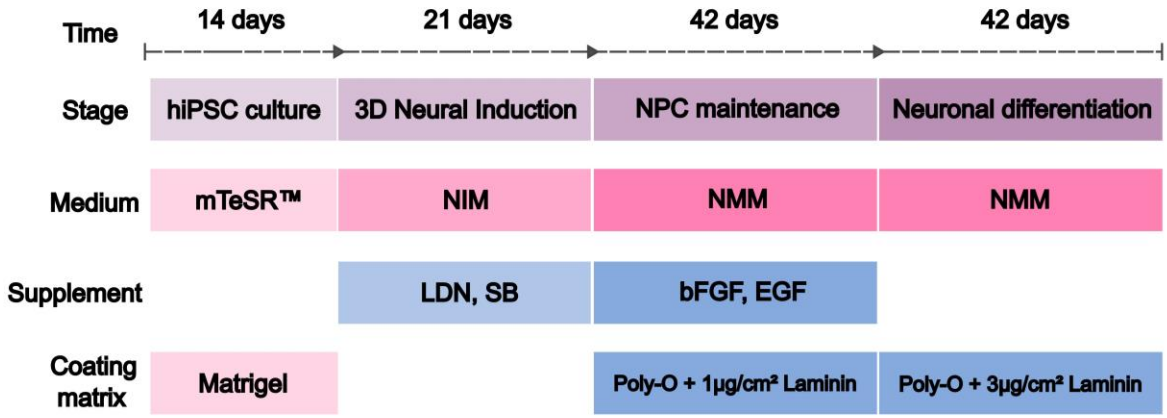

Figure 5. Schematic overview of the in vitro neuronal differentiation of hiPSCs demonstrating the timing of each stage, as well as the culture medium, supplements and coating matrices used. Poly-O-ornithine and Laminin were used for plate coating. LDN and SB, potent inhibitors of TGF-β/BMP/activin signalling pathways, were used for dual SMAD inhibtion to induce neuroectoderm formation. NIM (Neural induction medium), NMM (Neuronal maintenance medium), LDN (LDN193189), SB (SB431542 Hydrate), bFGF (Basic-fibroblast growth factor), EGF (Epidermal growth factor).

3.4 Neural differentiation of NPCs

Neuronal progenitor cells (NPCs) were plated on culture dishes coated with poly-L-ornithine (Cat#: P4957) /laminin (Cat#: L2020) 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, Cat#: P4458) supplemented with 10 ng/ml of EGF (Cat#: SRP3027) 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.

3.5 BPA Treated 3D Neural Induction

BPA was dissolved in DMSO (Cat#: 34869) 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.

3.6 Immunocytochemical staining

Fixation of 3D NSC spheroids was performed with 4% Paraformaldehyde (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 4′,6-diamidino-2-phenylindole (DAPI, Cat#: MBD0020) 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.

3.7 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.

3.8 RT-qPCR

16 spheroids were collected at each timepoint, and experiments were performed in triplicate (n=3). Both the RNeasy Plus Micro Kit (Qiagen, Cat#: 74004) and the RNeasy Plus Mini Kit (Qiagen, Cat#: 74104) 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, Cat#: K1641), in accordance with the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase (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 (ThermoFisher, Cat#: S4438), 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 fetal 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).

3.9 Cell viability and cytotoxicity testing

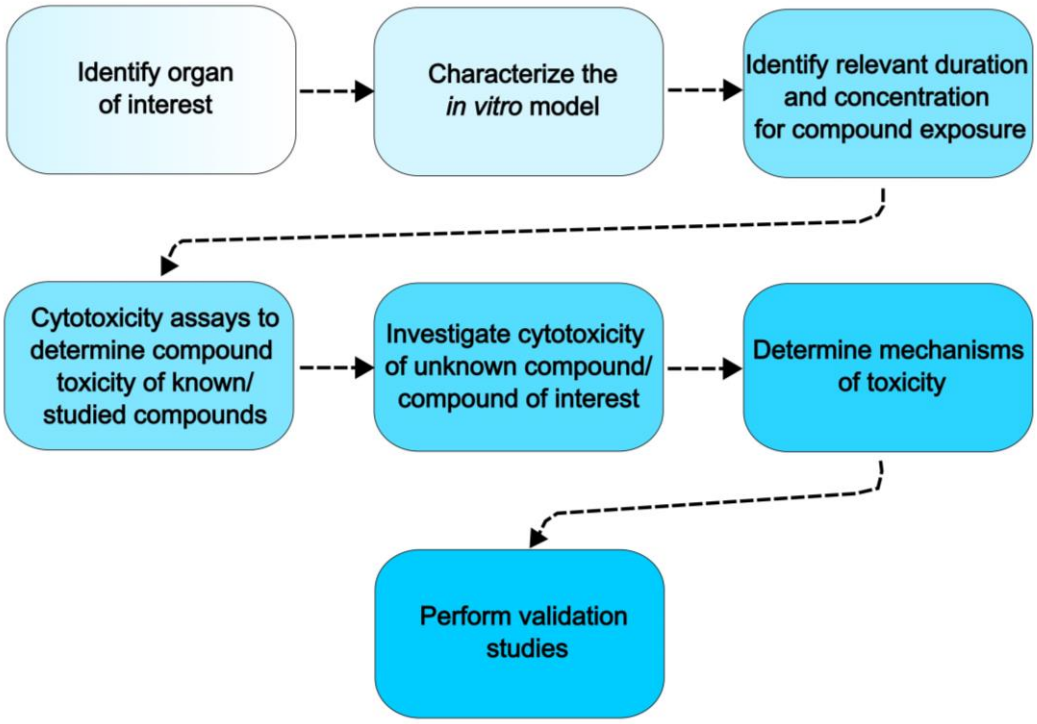

Figure 6. Overview of the workflow of the in vitro assessment of compound toxicity.

3.9.1 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, Cat#: G9681), 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.

3.9.2 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 lactate dehydrogenase (LDH) release. Using non-treated 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, Cat#: C20300), according to the manufacturer's instructions. Colourimetric signal was measured using a Thermo VarioScan Flash plate reader.

3.10 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.

3.11 Clonogenic assay

On day 21 of the BPA-treated neural induction, spheroids were dissociated using the neurosphere dissociation kit (Miltenyi Biotech), and re-plated at a single-cell density of 15,000 cells/well on Laminin coated 6-well plates. Cells were maintained in culture for a further 14 days, with media changes using Neuronal maintenance medium (NMM) supplemented with 5ng/mL bFGF and epidermal growth factor (EGF) every other day. At D14 after plating, plates were scanned using a flatbed scanner. ImageJ software and the countPHICS (count and Plot HIstograms of Colony Size) plugin were used to quantify the number of colonies formed in 0 µM 0.01µM, 0.1µM, and 1µM BPA treated groups. The survival fraction of BPA-treated NSCs was then calculated using the following formulas:

$$\textit{Plating efficiency} = \textit{number of counted colonies} / \textit{number of plated cells}$$

$$\textit{Surviving fraction} = (\textit{number of counted colonies} / \textit{number of plated cells}) / \textit{plating efficiency}$$

3.12 Analysis of ROS and mitochondrial levels

To measure reactive oxygen species (ROS) and mitochondrial levels of NSC spheroids at D21 of the BPA treated induction, spheroids were treated with 5 mM CellROX™ Deep Red (Invitrogen, Cat#: C10422) for 1h at 37°C or 100 nM MitoTracker™ Deep Red 633 (ThermoFisher, Cat#: M22426) for 30m at 37°C, respectively (ex/em; 630/650nm). Spheroids were then fixed for 1 hour at room temperature with 4% PFA in 0.1 mol/L phosphate buffer. Images were then acquired and the fluorescence signal was quantified using the Incucyte® Live Cell Analysis System (Sartorius, USA) (exposure time: 150ms). Spheroids that were being evaluated came from 3 independent experiments (n = 3).

3.13 Quantitative proteomics

3.13.1 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.

3.13.2 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.

3.14 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.

3.15 Network analysis

3.15.1 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 considered all proteins that were differently abundant for each condition, as well as breaking them down into up- and down-regulated proteins.

3.15.2 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.

3.15.3 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).

3.15.4 ALS candidate genes validation

Human post-mortem spinal cord gene expression count data were downloaded via the Zenodo platform (Humphrey et al., 2023). Further statistical analyses were conducted using the normalised Transcripts Per Kilobase Million (TPM) values of the cervical and lumbar regions, which are the two most relevant regions for ALS development. We used a two-tailed T test to assess the median expression of the genes that link ALS and the BPA-downregulated genes in both ALS patients and controls.

3.16 Ethical approval

The Hungarian Health Science Council's Scientific and Research Ethics Committee granted the ethical license in May 2021 for the "Production of Induced Pluripotent Stem Cells (IPS) from Human Somatic Samples" with the ID number IV/3935- 1 /2021/EKU.

4 RESULTS

4.1 hiPSC culture and characterization

To confirm the typical pluripotent characteristics of the SBAD2 hiPSC cell line we used a qualitative assessment of stem cell traits including colony shape and expression of key pluripotency markers.

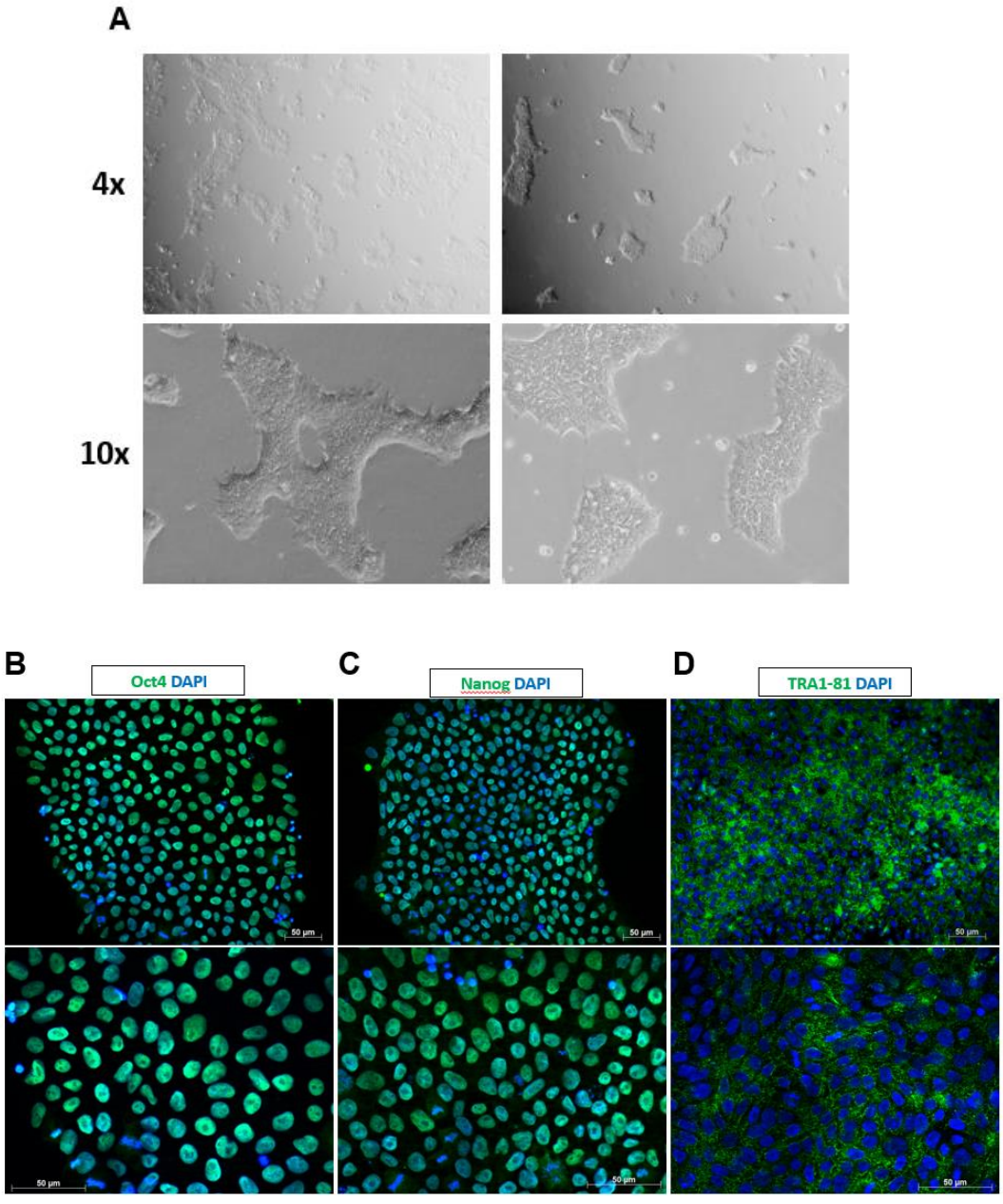

Figure 7. Characterization of SBAD hiPSCs in culture. (A) Phase contrast images of SBAD2 hiPSCs at x4 and x10 magnification. Pluripotency markers Oct4 (B), Nanog (C), and TRA1-81 (D) were stained in 2D The used fluorophore was Alexa 488 (green) Nuclei were counterstained with DAPI (blue). Scale bar, 50 µm.

We observed that hiPSCs formed distinctive colonies (Figure 7A), while immunocytochemical detection revealed that the hiPSCs expressed classical pluripotency markers Oct, Nanog and TRA1-81 (Figure 7B-D), thus confirming their pluripotent status in our in vitro setting.

4.2 Characterization of the neural induction of hiPSCs

4.2.1 2D Neural Induction

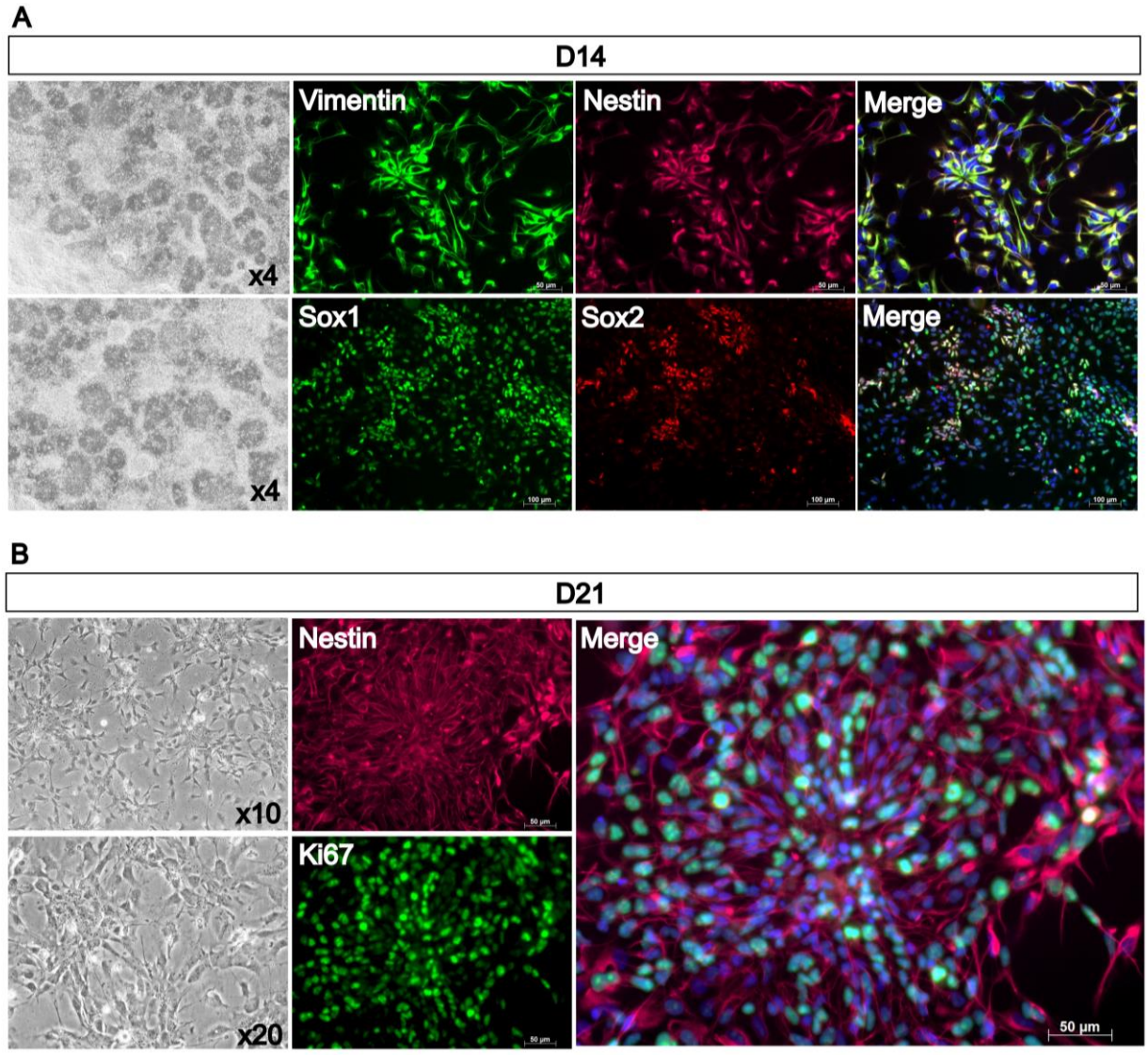

Figure 8. Immunostaining of the 2D neural induction. (A) Phase contrast images at D14 of the 2D neural induction of SBAD2 hiPSCs at x4 magnification and immunocytochemical staining of key neural lineage and NSC markers Nestin, Vimentin, Sox1 and Sox2. (B) Phase contrast images at D21 of the 2D neural induction of SBAD2 hiPSCs at x4 and x10 magnification, and immunocytochemical staining of key NSC marker Nestin, and proliferation marker Ki67. The used fluorophores were Alexa 488 (green) or Alexa 546 (Red). Nuclei were counterstained with DAPI (blue). Scale bars are displayed.

To qualitatively characterise the 2D neural induction of hiPSCs to NSCs, we performed immunocytochemical labelling at D14 and D21 of the induction. The positive expression of key neural lineage and neuroectodermal markers including Nestin, Sox1, Sox2 and Vimentin, as well as the presence of key proliferation marker Ki67 (Figures 8A, B) suggests a successful differentiation to NSC stage. Additionally, brightfield images demonstrated that the differentiating cultures displayed a neural rosette morphology typical of neuroepithelial cells.

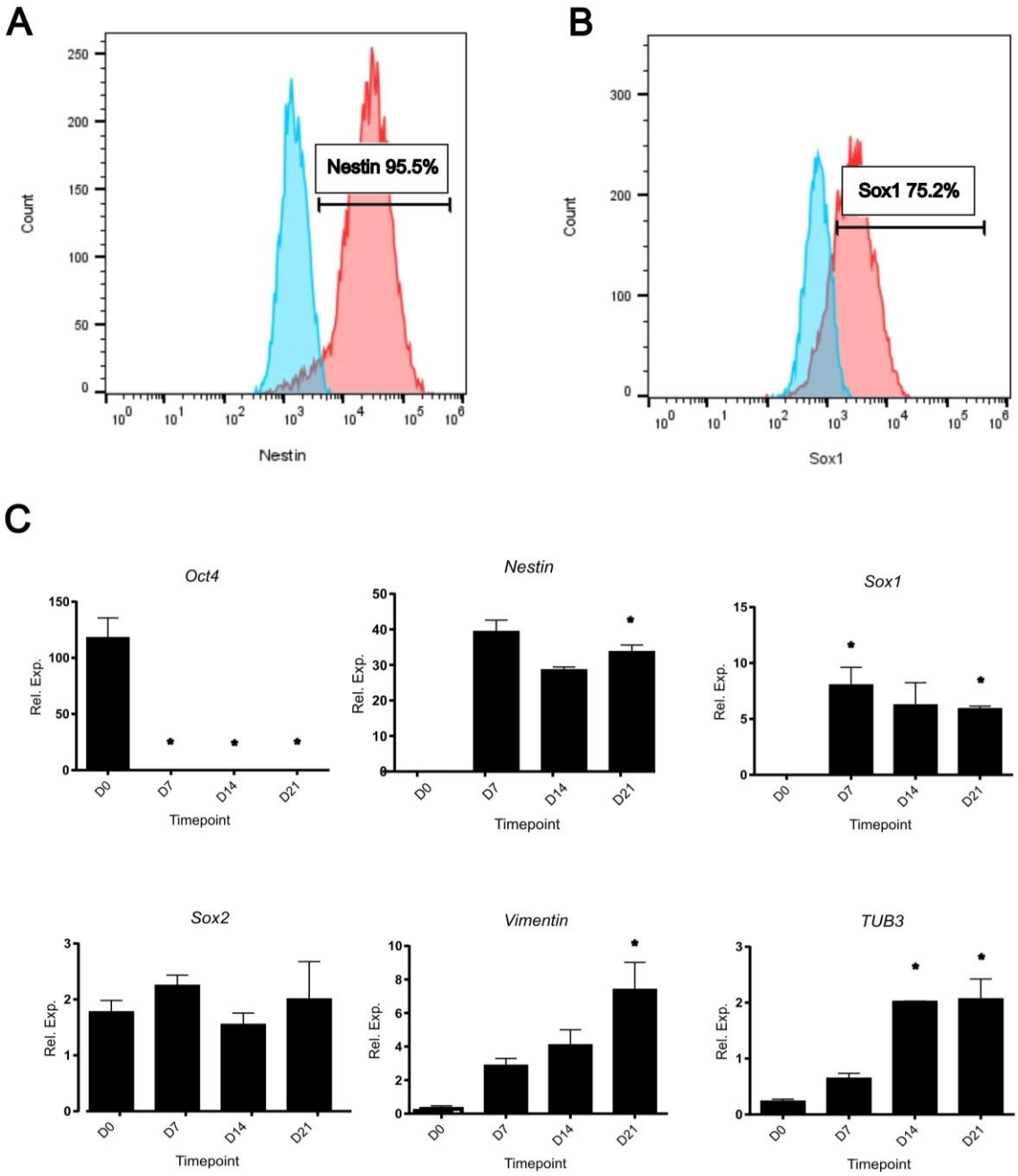

Figure 9. Characterization of the 2D Neural Induction via RT-qPCR and Flow Cytometry. Flow cytometry representation of hiPSC-derived NSCs from the 2D neural induction at D21 of the differentiation for (A) Nestin and (B) Sox1. Light blue peak represents the unstained control. (C) Weekly real-time qPCR measurements of the 2D neural induction of hiPSCs. Graphs represent normalised relative expression

values. GAPDH was used as a reference gene and data was normalised using Takara human cortical RNA. The results shown are from 3 independent experiments ($n = 3$). One-way ANOVA with Dunnett's post hoc test was used to determine significance (adjusted p-value * $p < 0.05$). ± SEM are displayed.

For a deeper characterization of the 2D neural induction, we performed flow cytometry and RT-qPCR analysis. Using flow cytometry we assessed the efficiency of the differentiation, with 95.5% and 75.2% of cells positive for Nestin and Sox1, respectively by day 21 (Figure 9A, B). Using RT-qPCR, we observed significant increases to the transcript levels of classical neural lineage and neuroectodermal markers Nestin, Sox1, Vimentin and Tub3 by day 21 of the 2D neural induction (Figure 9C). Additionally, we observed a decrease to the mRNA level of pluripotency marker Oct4 by day 7 of the differentiation.

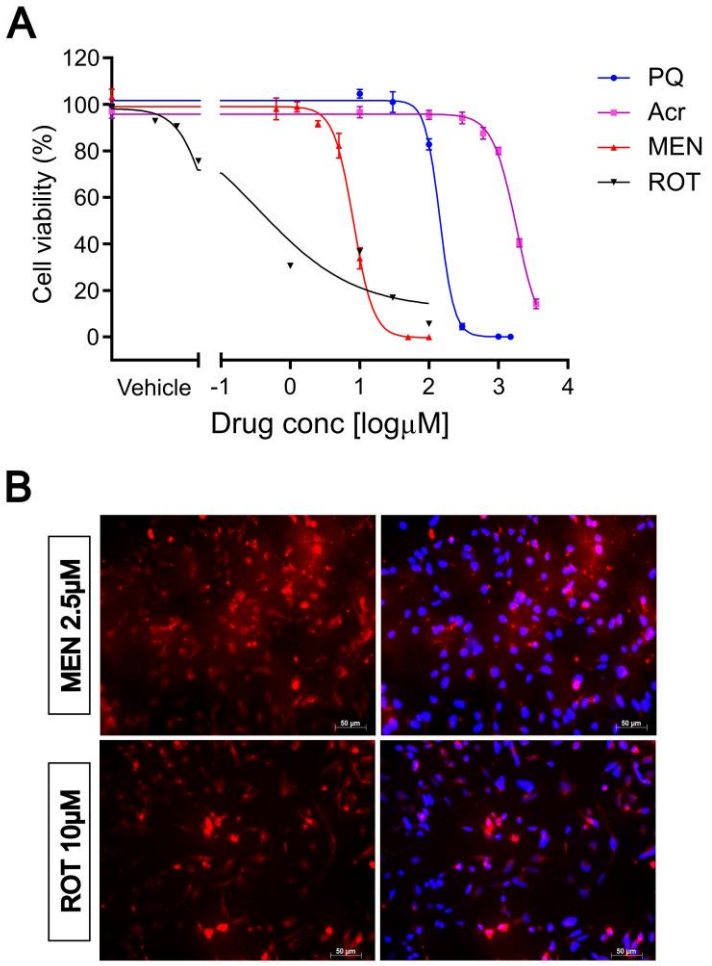

Figure 10. Assessing compound viability and ROS induction in 2D NSCs. (A) displaying cell viability results obtained using the Cell-titer Glo Luminescent Cell Viability Assay. SBAD2 2D NSCs were treated with increasing concentrations of Paraquat (PQ), Acrylamide (Acr), Menadione (MEN) and Rotenone (ROT) for 48hrs. Standard error bars are shown. (B) CellROX Deep Red ROS detection after 48-hour treatment with Men (2.5uM), and Rot (1uM).

To determine sub-cytotoxic ranges of ROS-inducing compounds and investigate the suitability of our 2D NSC cultures for assessing the toxicity of environmental chemicals on the developing brain in vitro, we performed ATP assays to generate dose-response curves for Paraquat, Menadione, Rotenone and Acrylamide (Figure 10A). We demonstrated that our model produced repeatable dose-responses that can be used for accurately modelling compound cytotoxicity in vitro. In addition, we used the CellROX assay to perform a qualitative detection of ROS production from well-known ROS-inducing compounds Rotenone and Menadione, observing a positive fluorescent signal which indicated the presence of ROS in culture upon treatment with these compounds (Figure 10B).

4.2.2 3D Neural Induction

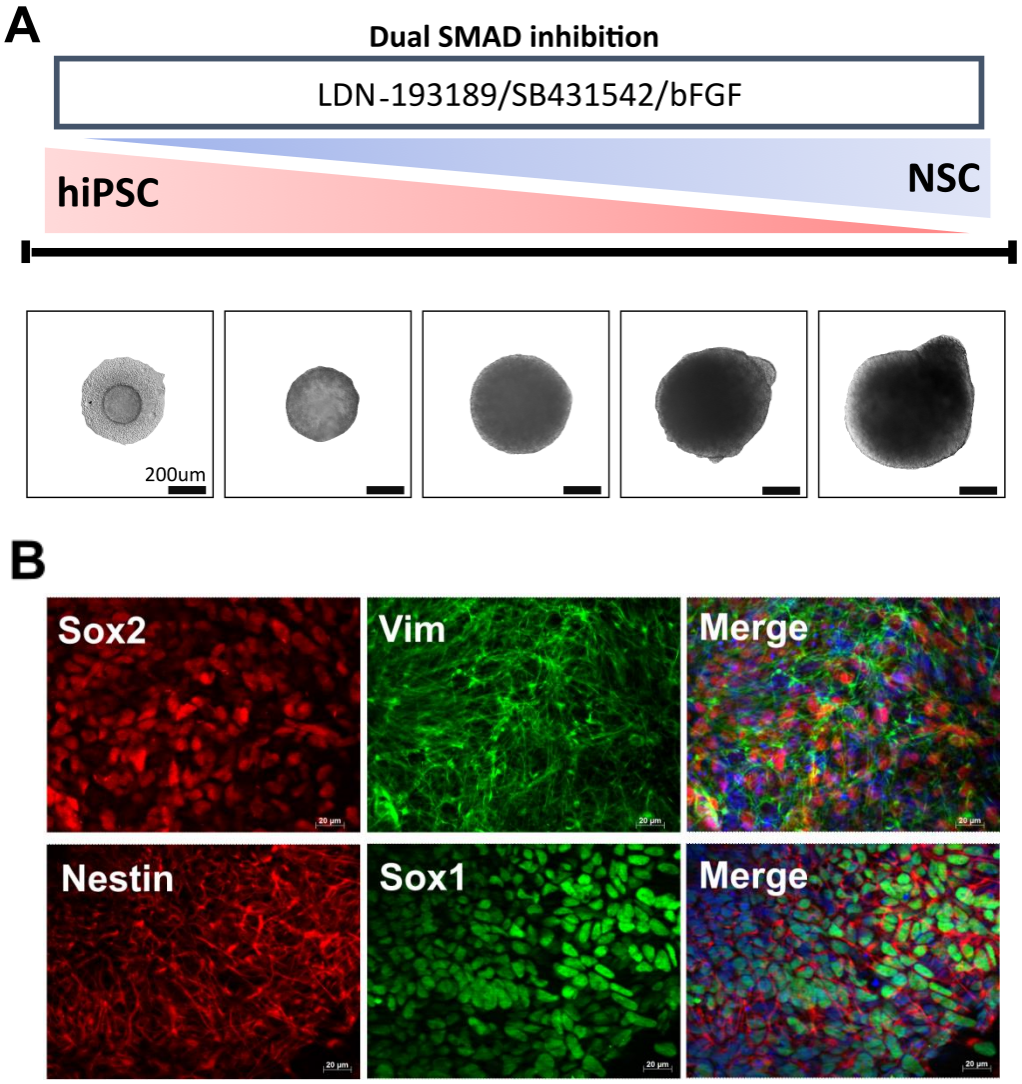

Figure 11. Characterization of hiPSC-derived NSCs. (A) Schematic overview and phase contrast images of the 3D in vitro neural induction of hiPSCs to NSCs. (B) Immunocytochemical detection of spheroids at D21 of the in vitro neural induction of hiPSCs. Neural stem cell and neuroectodermal proteins Vimentin,

Sox2, Nestin and Sox1 were stained in 3D culture. Fluorophores used were Alexa 488 (green) and Alexa 594 (red). Nuclei were counterstained with DAPI (blue).

To assess the suitability of the 3D neural induction to model NSC differentiation in vitro, we characterised hiPSC-derived NSCs. After beginning the 3-week neural induction with the dual-SMAD inhibition method, hiPSC spheroids were successfully formed after 48 hours. In addition, phase contrast images showed a steady growth of spheroid size throughout the duration of the differentiation (Figure 11A). ICC detection was also used to highlight the expression of Sox1, Sox2, Nestin and Vimentin in NSC spheroids at day 21 of the neural induction (Figure 11B).

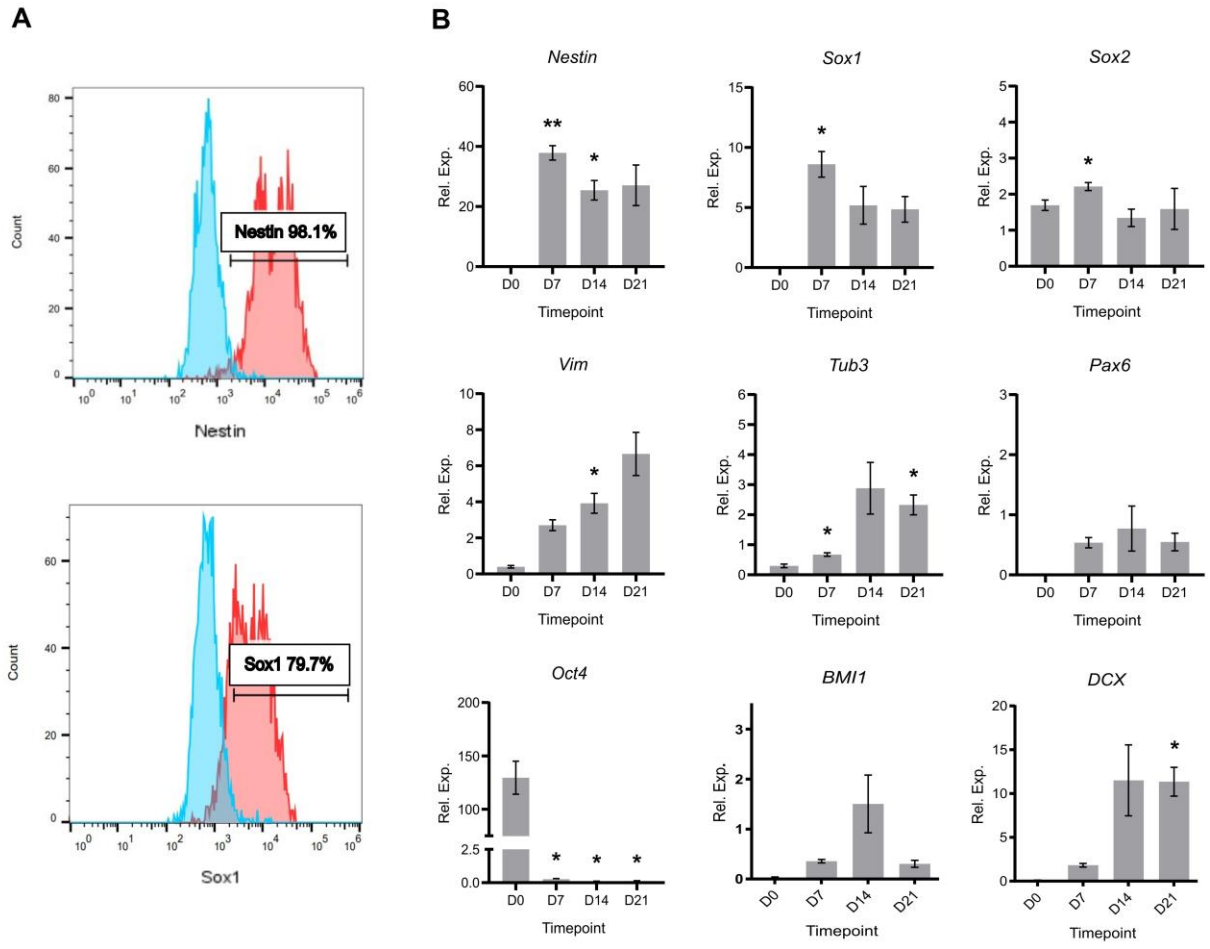

Figure 12. Characterization of the 3D Neural Induction via RT-qPCR and Flow Cytometry. (A) Flow cytometry representation of hiPSC-derived NSC spheroids from the 3D neural induction at D21 of the differentiation for Nestin and Sox1. Light blue peak represents the unstained control. (B) Weekly real-time qPCR measurements of the 3D neural induction of hiPSCs. Graphs represent normalised relative expression values. GAPDH was used as a reference gene and data was normalised using Takara human cortical RNA. The results shown are from 3 independent experiments ($n = 3$). One-way ANOVA with Dunnett's post hoc test was used to determine significance (adjusted p-value * $p < 0.05$, ** $p < 0.01$). ± SEM are displayed.

To further characterise the 3D neural induction, we performed flow cytometry and RT-qPCR analysis. Using flow cytometry analysis, we assessed the efficiency of the hiPSC to NSC differentiation by measuring the percentage of cells that expressed key neuroepithelial markers. We observed 98.1% and 79.7% of cells positive for Nestin and Sox1, respectively by day 21 of the 3D neural induction (Figure 12A). Additionally, we measured the expression levels of key NSC transcripts throughout the duration of the neural induction using RT-qPCR (Figure 12B). 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.

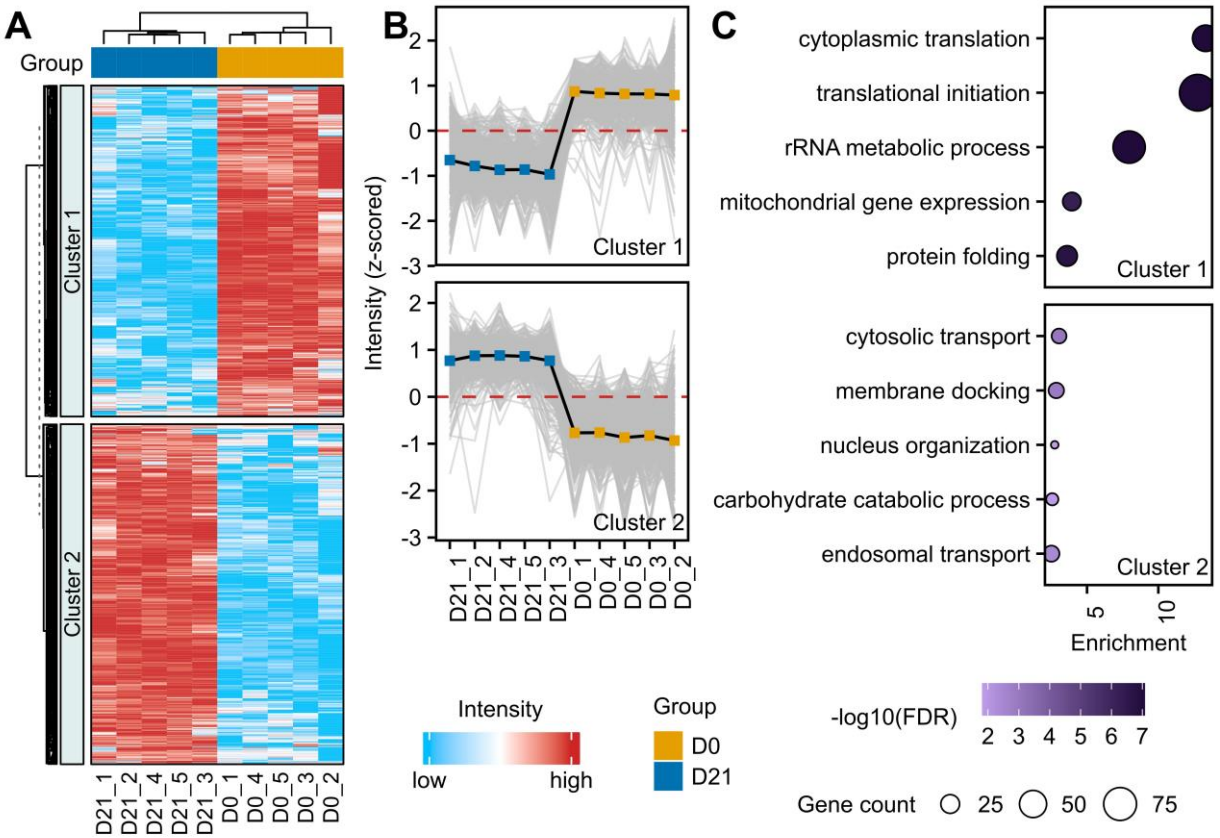

Figure 13. Proteome alteration upon 3D neural induction of hiPSCs. (A) Unsupervised hierarchical clustering of significantly altered proteins (fold change > 1.3, adjusted *p*-value < 0.05) when comparing D21 to D0 ($n = 5$). For standardised protein abundances, red represents those in high abundance, while blue represents those in low abundance. To partition heatmap rows into homogenous regions, k means algorithm was used ($k = 2$). (B) Profile plots displaying mean values of two clusters showing protein changes between day 0 (hiPSC) and day 21 (NSC). (C) Protein enrichment analysis from each cluster. Bubble size corresponds to the number of differentially abundant proteins (referenced as gene count within the figure).

Bubble colour corresponds to the significance of the enrichment. Enrichment shows the magnitude of over representation.

For a more in-depth characterization of NSCs derived from the neural induction of hiPSCs, we 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) (Figure 13A-C). With high confidence (false-discovery rate <0.01) 4733 proteins were identified. 60 % of the quantified were altered at day 21 compared to day 0. There were increases to proteins involved with membrane docking, cytosolic transport and nuclear organisation, while there were decreases to proteins related to cytoplasmic translation, translational initiation and rRNA metabolic processes (Figure 13C). 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 (Figure 12). On the other hand, pluripotency markers LIN28A and Oct-4 ere decreased in abundance in NSCs compared to hiPSCs.

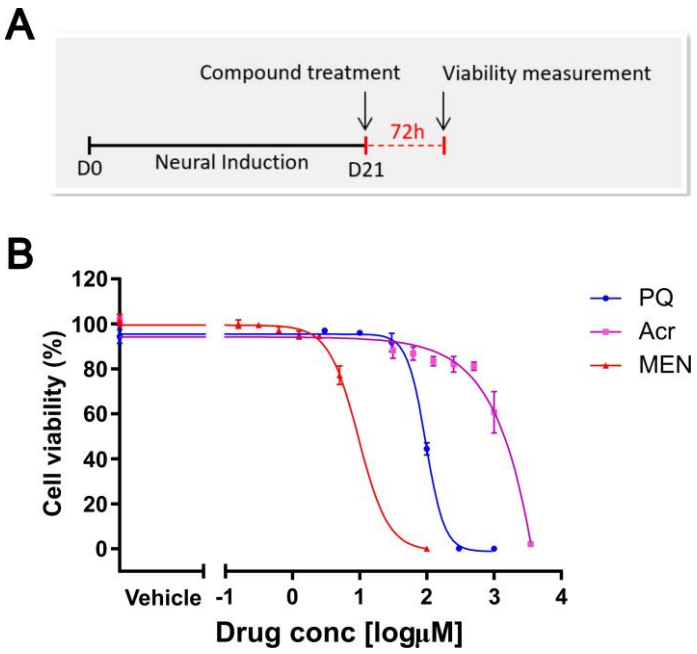

Figure 14. Assessing compound cytotoxicity in 3D NSC spheroids. (A) Schematic overview of 48-hour compound treatment and viability measurements using 3D NSC spheroids. (B) Cell viability results obtained using the Cell-titer Glo Luminescent Cell Viability Assay. 3D NSC spheroids were treated with increasing concentrations of Paraquat (PQ), Acrylamide (Acr), and Menadione (MEN) for 48hrs. Standard error bars are shown (n =3).

To determine sub-cytotoxic ranges of environmental chemicals and investigate the suitability of our 3D NSC cultures for assessing the toxicity of environmental chemicals on the developing brain in vitro, we performed ATP assays to generate dose-response curves for Paraquat, Menadione and Acrylamide. We demonstrated that our model produced repeatable dose-responses that can be used for accurately modelling environmental chemical cytotoxicity in vitro, and for further neurotoxicological applications including DOHaD-related investigations (Figure 14 A, B).

4.3 Neuronal differentiation of NPCs

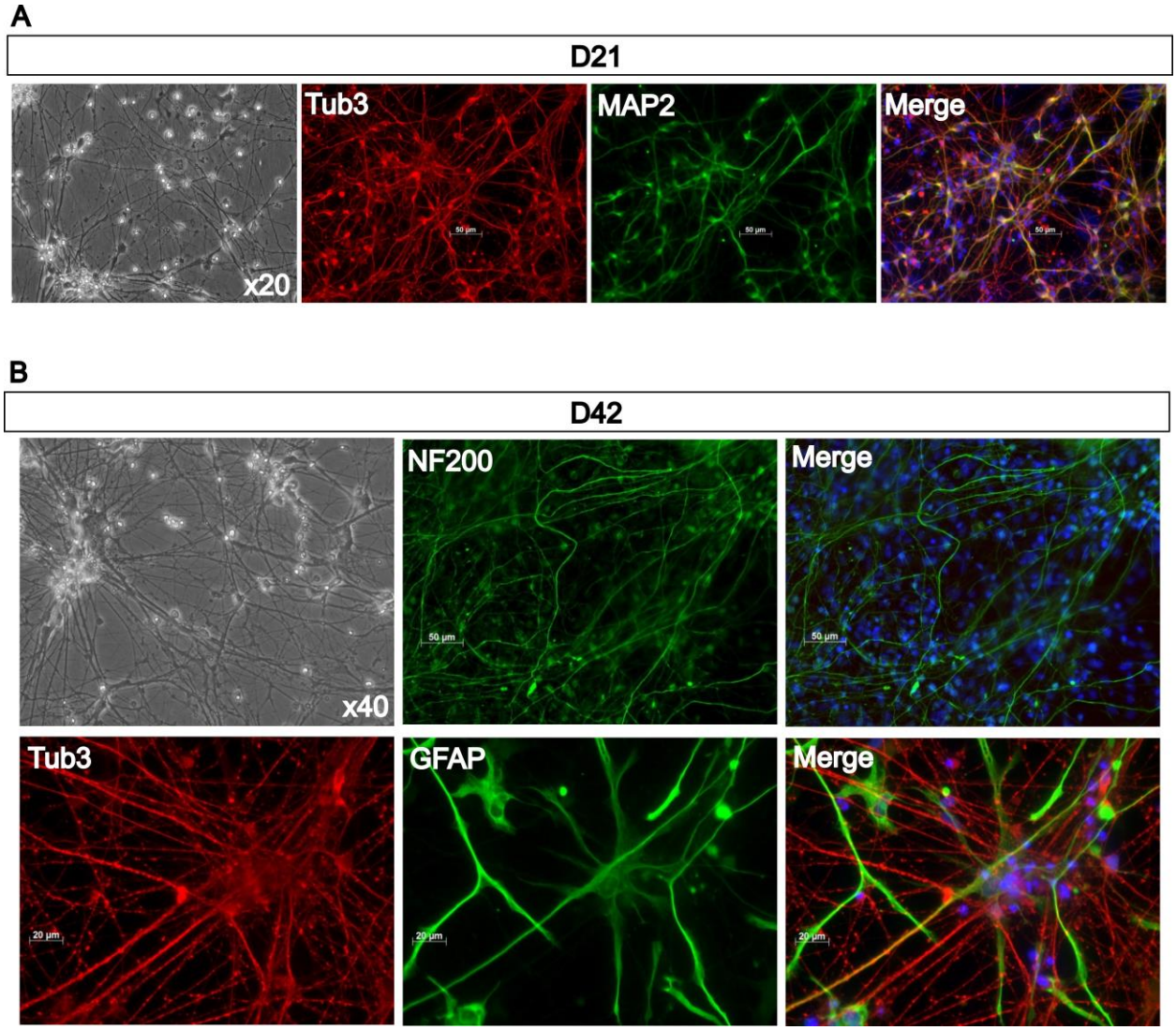

Figure 15. Immunostaining of the 2D terminal neuronal differentiation. (A) Phase contrast images at day 21 of the 2D terminal neuronal differentiation of SBAD2 NPCs at x20 magnification and immunocytochemical staining of key neuronal markers Tub3 and MAP2. (B) Phase contrast images at day 42 of the 2D terminal neuronal differentiation at x40 magnification, and immunocytochemical staining of neuronal markers NF200 and Tub3, as well as astroglia-specific marker GFAP. The used fluorophores were

Alexa 488 (green) or Alexa 546 (Red). Nuclei were counterstained with DAPI (blue). Scale bars are displayed.

To further demonstrate the strength of our model for in vitro neural differentiation, we performed the terminal neuronal differentiation of obtained NSCs. Over 42 days, we obtained mixed cultures of cortical neurons and glia cells in vitro. By day 21 of the terminal neuronal differentiation, we observed the expression of immature neuronal marker Tub3, as well as mature neuronal marker MAP2 via immunocytochemical staining (Figure 15A). Furthermore, by day 42 of the neuronal differentiation, cells expressed neuronal protein NF200, as well neuroglial marker GFAP which demonstrated the presence of astrocytes in culture (Figure 15B).

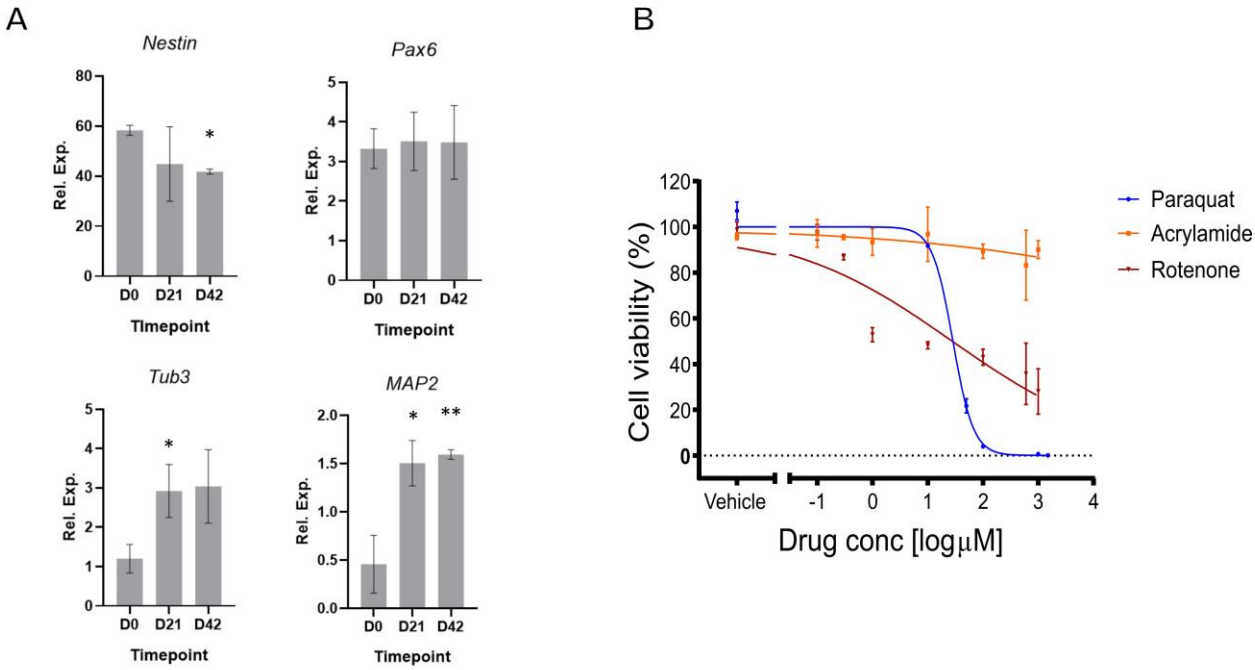

Figure 16. Gene expression and cytotoxicity analysis of terminally differentiated neuronal cultures. (A) Real-time qPCR measurements of the 2D terminal neuronal differentiation. Graphs represent normalised relative expression values. GAPDH was used as a reference gene and data was normalised using Takara human cortical RNA. The results shown are from 3 independent experiments (n = 3). One-way ANOVA with Dunnett's post hoc test was used to determine significance (adjusted p-value * $p < 0.05$, ** $p < 0.01$). ± SEM are displayed. (B) Cell viability results obtained using the Cell-titer Glo Luminescent Cell Viability Assay. Neuronal cultures at day 21 were treated with increasing concentrations of Paraquat (PQ), Acrylamide (Acr), and Rotenone (ROT) for 48hrs (n = 3). Standard error bars are shown.

By day 21 of the terminal neuronal differentiation, there was a significant increase to the transcript expression levels of Tub3 and MAP2, and by day 42 there was a significant decrease in the mRNA levels of NSC/neuroepithelial marker Nestin (Figure 16A). The transcript and protein expression levels of neuronal markers during the in vitro terminal neuronal differentiation (Figures 15, 16)

highlighted that our system provides a suitable platform for modelling embryonic brain development from the early stages of neurulation through to neurogenesis. To examine the capacity of the terminally differentiated neuronal cultures for neurotoxicological screening and DOHaD-related investigations, we performed ATP viability assays with increasing concentrations of environmental chemicals Paraquat, Acrylamide and Rotenone (Figure 16B). We demonstrated that our model facilitated the generation of repeatable dose-response curves and is therefore suitable for further in vitro neurotoxicological assessments.

4.4 BPA treated neural induction of hiPSCs

4.4.1 Viability analysis

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 (Figure 17A). 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 (Figure 17B).

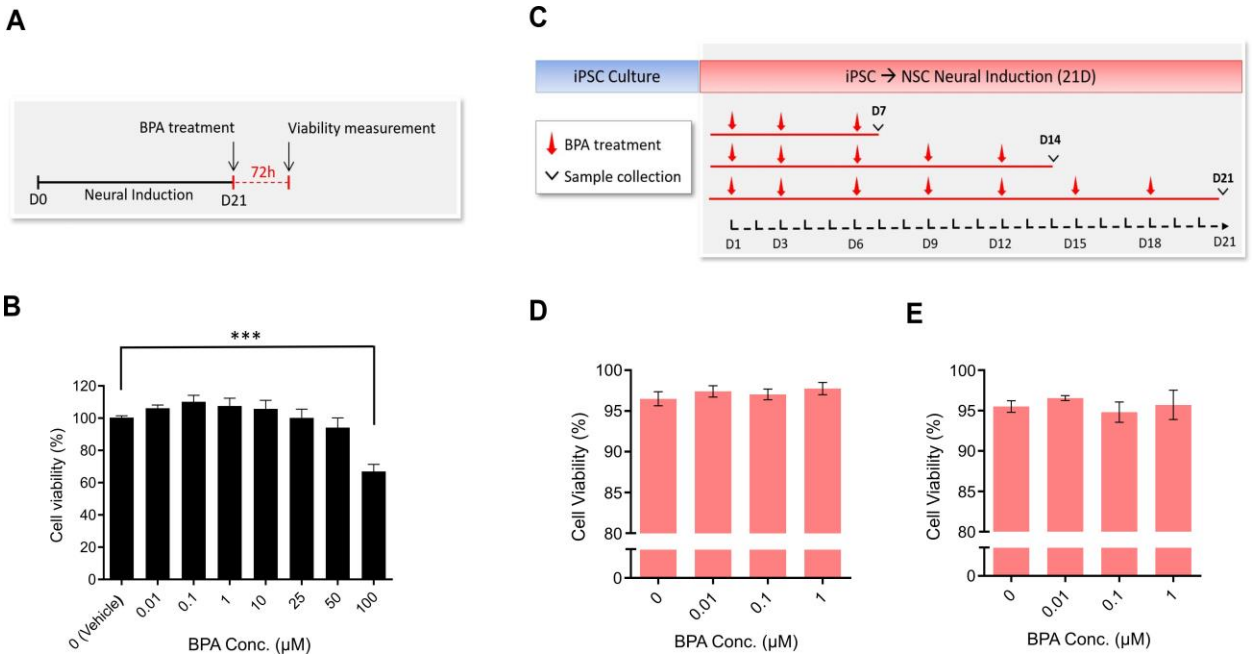

Figure 17. Cell viability analysis of NSC spheroids after BPA treatment. (A) Schematic outline and **(B)** dose-response representing the cell viability (%) of NSC spheroids (n = 3) after 72-hour BPA exposure. **(C)** Experimental schematic of the repeated-dose BPA exposure during the neural induction of hiPSCs over 21 days, and LDH assay demonstrating viability (%) determined from LDH release of NSC

spheroids at **(D)** D14 (n = 3) and **(E)** D21 (n = 3) of the BPA treated neural induction. One-way ANOVA with Dunnett's post hoc test was used to determine significance (adjusted p-value *** $p < 0.001$) ± SEM are displayed.

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 (Figure 17C). 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 (Figure 17D, E) or day 21 of the BPA-treated neural induction, compared to vehicle-treated controls.

4.4.2 Repeated-dose BPA exposure affects NSC spheroid growth.

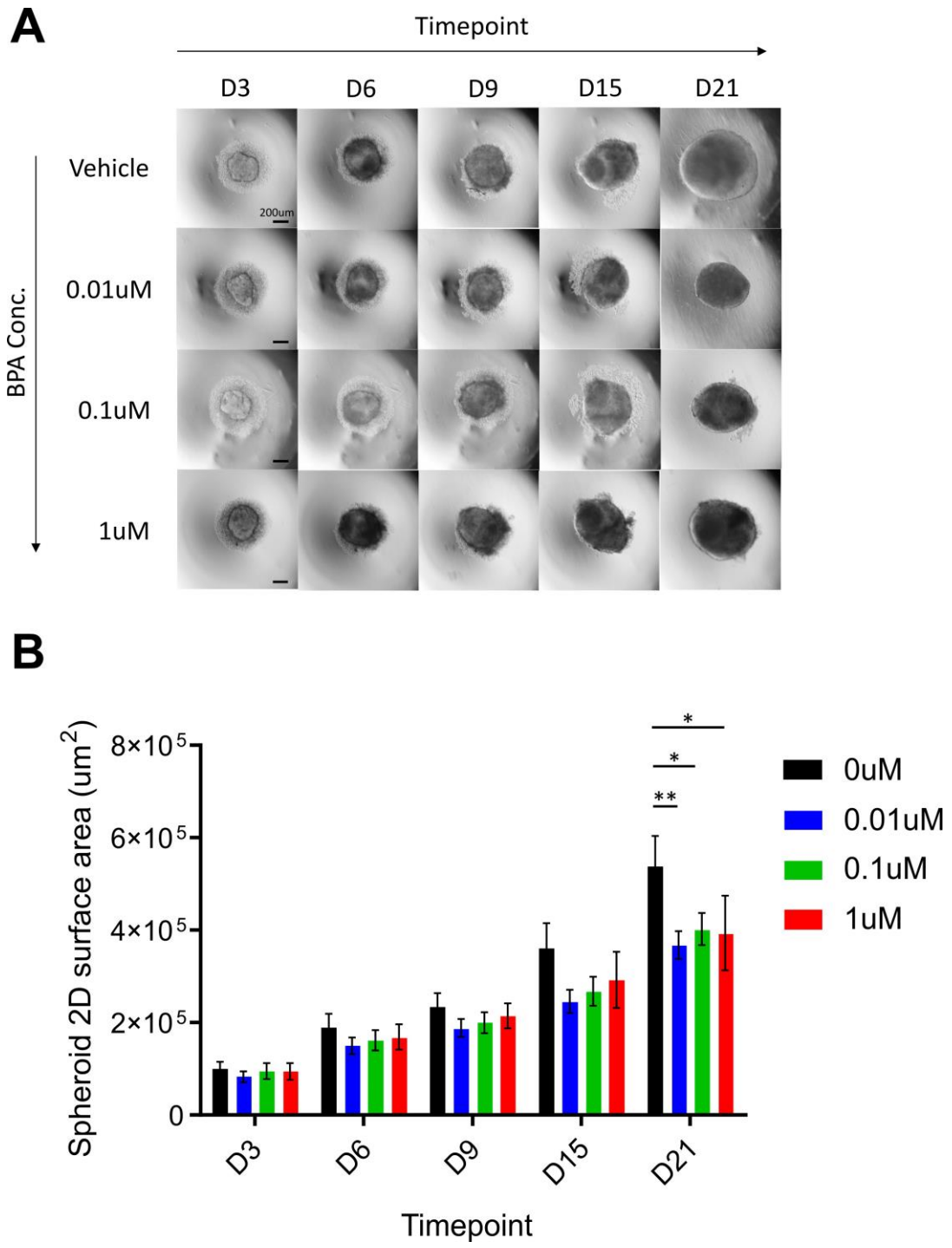

Figure 18. Microscopic aspect and growth curves of NSC spheroids during the BPA-treated neural induction. (A) Microscopic aspect of spheroids using the x4 objective (0.1 NA) of Olympus IX71 microscope and DP21 camera. Scale bar, 200µM. (B) NSC spheroid growth monitored for 21 days over the duration of the BPA-treated neural induction of hiPSCs. Spheroids were treated with repeated doses of 0µM, 0.01µM, 0.1µM or 1µM BPA. The 0µM (Vehicle) group was treated with 0.1% DMSO. Results were obtained from 4 biological replicates (n=4) where 8 spheroids were measured in each group. Two-way

ANOVA with Dunnett's post hoc test was used to determine significance (adjusted *p*-value * $p < 0.05$, ** $p < 0.01$). ± SEM are displayed.

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 (Figure 18A). 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 (Figure 18B).

4.4.3 Effects of BPA exposure on ROS and mitochondrial levels in NSCs

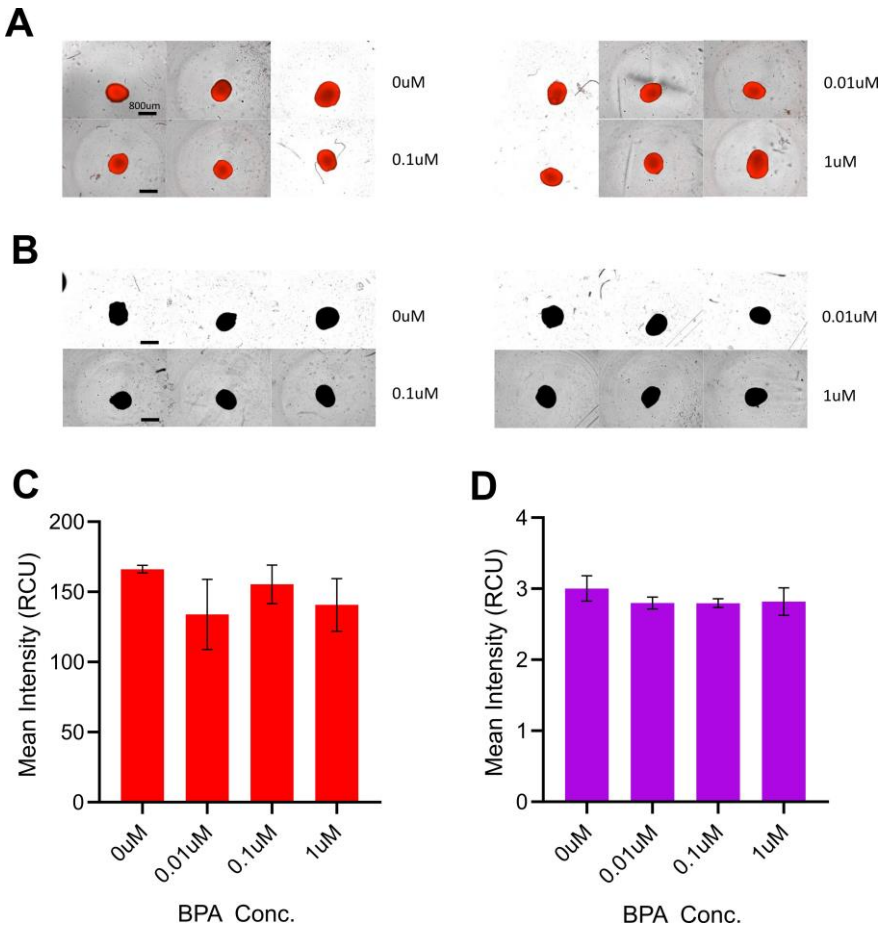

Figure 19. Fluorometric image analysis of ROS and mitochondrial levels at day 21 of the BPA-treated neural induction. Incucyte® Live Cell Analysis System was used to capture images of day 21 NSC spheroids for analysis of (A) mitochondrial and (B) ROS levels. Scale bar, 800µm. Average fluorescence measurements for (C) mitochondrial levels and (D) ROS levels of spheroids at day 21 of the BPA-treated neural induction. Incucyte® Live Cell Analysis System was used to quantify image fluorescence. 0µM

groups were treated with vehicle only (0.1% DMSO). Three biological replicates were performed (n=3). ±SEM is displayed. One-way ANOVA with Dunnett's post hoc test was used to determine significance.

To determine the effects that the repeated-dose BPA exposure had on mitochondrial levels in NSCs, we used the MitoTracker Deep Red fluorometric assay, widely used to stain mitochondria in viable cells. No significant alterations to the mean fluorescence signal were observed in BPA-treated groups compared the vehicle-treated control group at day 21 of the neural induction, which implied that there were no changes to mitochondrial levels in BPA treated NSCs (Figure 19C). Additionally, we investigated the effects that BPA treatment had on cellular ROS levels with the CellROX™ Deep red fluorometric assay. No significant change to the average fluorescence levels were detected in BPA treated spheroids, suggesting that BPA treatment at these concentrations did not affect NSC ROS levels (Figure 19D).

4.4.4 Effects of BPA exposure on NSC clonogenicity

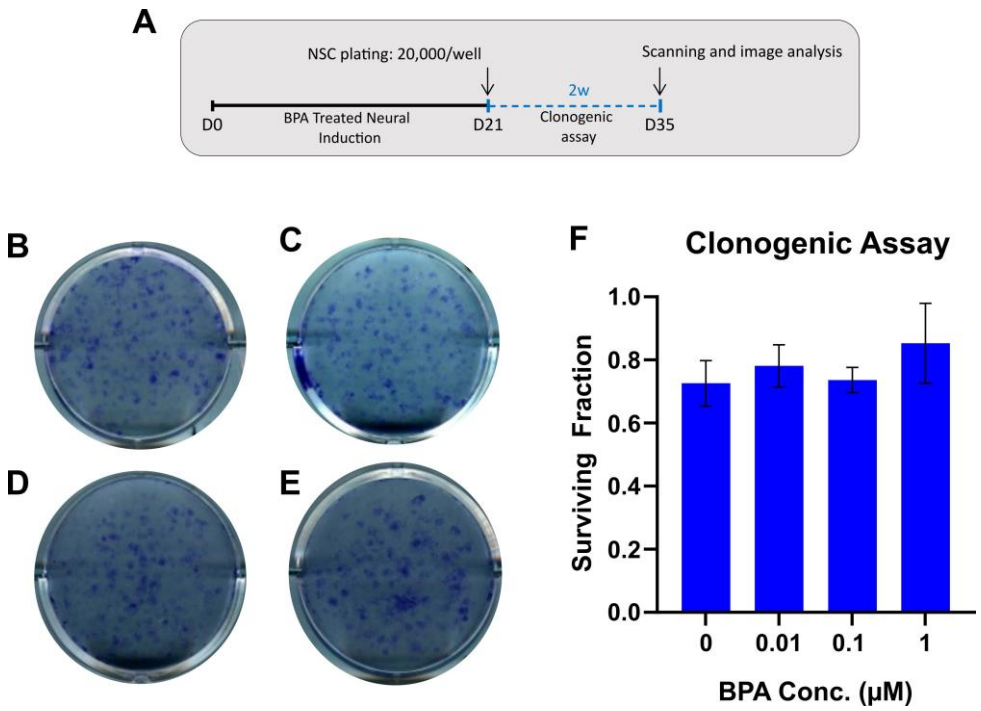

Figure 20. Clonogenicity measurements of BPA-treated NSCs. Clonogenic assay performed in 6-well plate format with NSCs obtained from the BPA treated neural induction. (A) Schematic overview and scanned representative images of (B) vehicle-treated control, (C) 0.01µM treated group, (D) 0.1µM treated group and (E) 1µM treated group. (F) Survival curves of the clonogenic survival assays. Survival fractions indicate the ratio of the plating efficacy of the BPA-treated NSCs to the non-treated NSCs. Cells were treated with the displayed concentrations of BPA during the neural induction for 21 days, then re-plated in 6-well plates. The colonies were counted at 14 days post-BPA treatment.

To assess the effects that BPA exposure exerted on the colony forming capacity of NSCs, we performed a clonogenic assay to measure the number of colonies formed from single cells in vehicle-treated and BPA-treated groups. No significant alterations to the surviving fraction were detected in BPA-treated groups compared to the control group, indicating that there was no change to the clonogenic potential in BPA-treated NSCs (Figure 20F).

4.4.5 Effects of BPA exposure on NSC mRNA transcripts

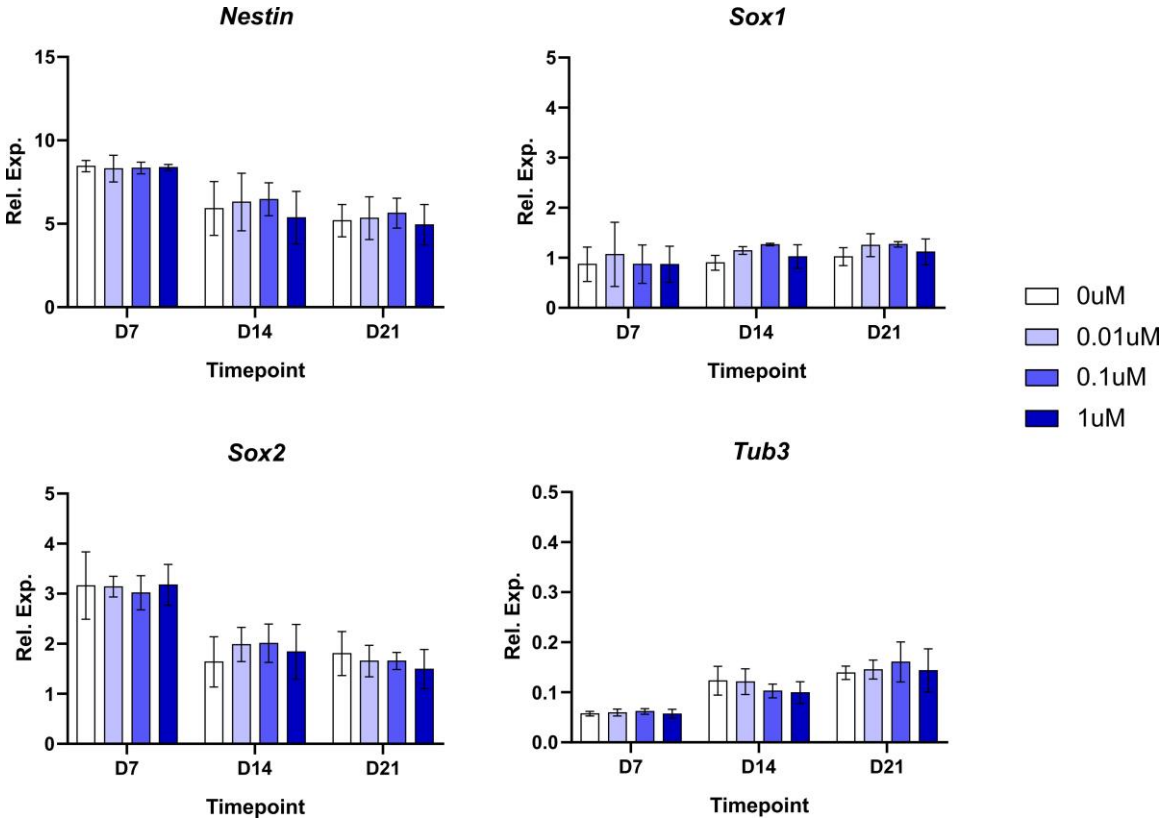

Figure 21. RT-qPCR measurements of spheroids at weekly intervals during the BPA-treated neural induction. GAPDH was used as a reference gene and normalised relative expression values are shown. Data normalization was carried out using Takara fetal brain RNA and 3 biological replicates were measured (n = 3). Significance was determined using Two-Way ANOVA with Dunnett's post hoc test ± SEM are displayed.

To investigate BPA-induced alterations to vital NSC transcripts and potential changes to the hiPSC to NSC differentiation rate, we performed weekly measurements of gene expression using RT-qPCR during the BPA-treated 3D neural induction (Figure 21). The relative expression of key neuroectodermal markers Sox1 and Nestin, as well as key neural lineage markers Tub3 and Sox2 were assessed. No significant changes in the mRNA levels of these genes were observed at all

timepoints after BPA exposure, which suggested that there was no alteration to the hiPSC to NSC differentiation rate in BPA-treated groups compared to the vehicle-treated control group.

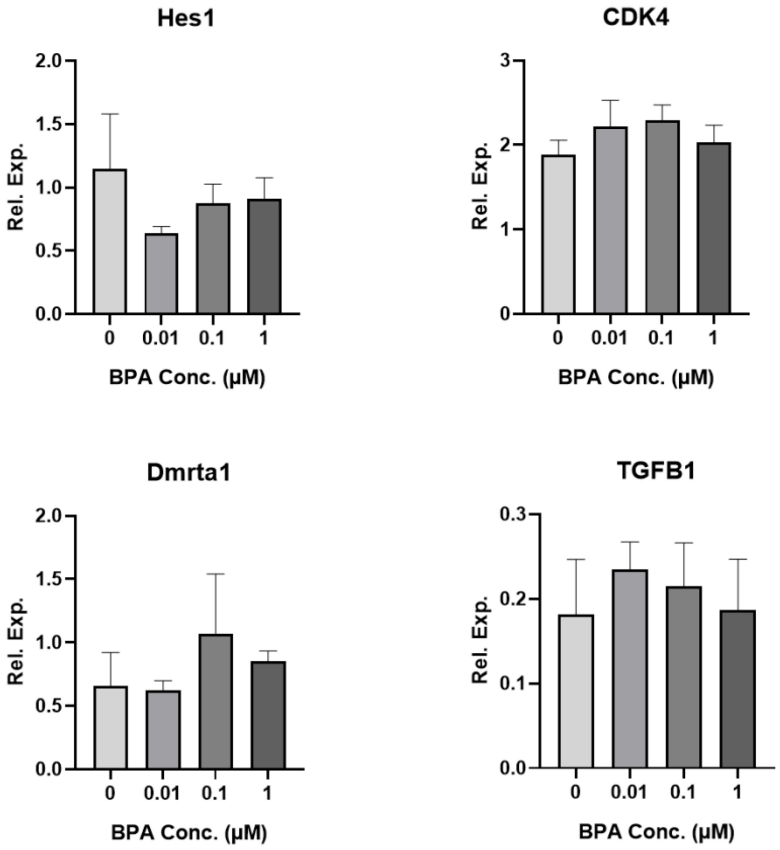

Figure 22. RT-qPCR measurements of spheroids at day 21 of the BPA-treated neural induction. GAPDH was used as a reference gene and normalised relative expression values are shown. Data normalization was carried out using Takara fetal brain RNA and 3 biological replicates were measured (*n* = 3). Significance was determined using Two-Way ANOVA with Dunnett's post hoc test ± SEM are displayed.

To investigate the potential mechanisms underlying the altered spheroid size at day 21 of the BPA treated neural induction, we measured the mRNA levels of genes with critical roles in the regulation of NSC maintenance and proliferation (Figure 22). At day 21 of the BPA-treated neural induction, there was no significant change to the relative expression of Hes1, CDK4, Dmrta1 or TGFβ1.

4.4.6 Proteome alterations in BPA-treated NSCs

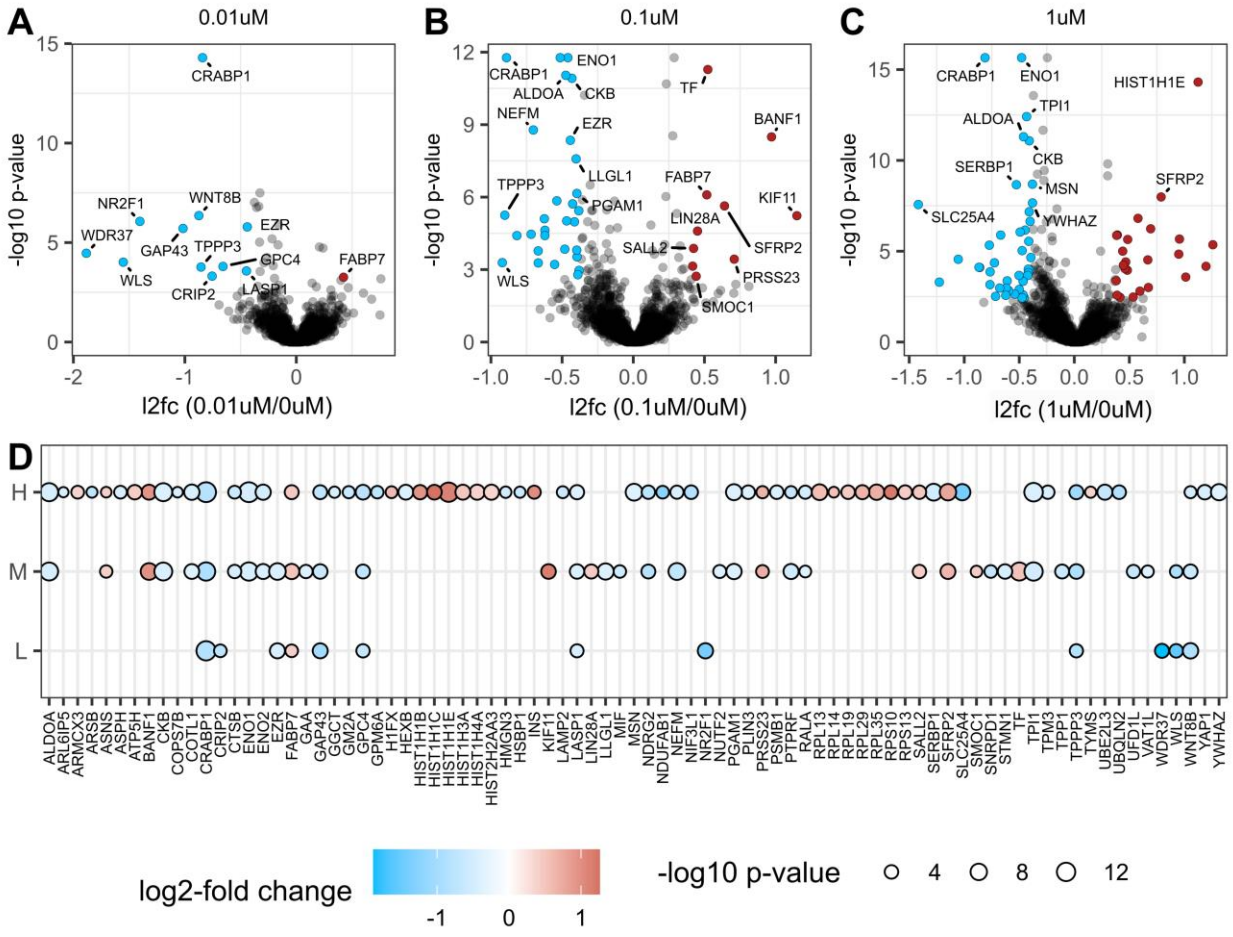

Figure 23. Proteome analysis at day 21 of the BPA-treated 3D neural induction. Proteome changes represented by volcano plots in NSCs treated with (A) 0.01µM, (B) 0.1µM and (C) 1µM BPA over the course of the 3D neural induction of hiPSCs. 4 biological replicates were measured for each group (n = 4). (D) Significantly changed proteins in at least one comparison are displayed on a bubble plot (adjusted p-value <0.05 and fold change <1.3); L (0.01µM/D21), M (0.1µM/D21), H (1µM/D21). Log2 fold change of the proteins is represented by circle colour (upregulated in red, downregulated in blue), while circle size is representative of the Benjamini-Hochberg-adjusted-log10 p-value. L, low concentration; M, middle concentration; H, high concentration. 0µM represents the vehicle-treated control.

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 (Figure 17C). 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 (Figure 23A-D). 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. No significant alterations were observed to the abundances of key NSC proteins Sox1, Nestin, Tub3, Sox2, which supports our previous RT-qPCR findings (Figure 21). 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 fetal brain.

4.4.7 Protein and disease network analysis of BPA-treated NSCs

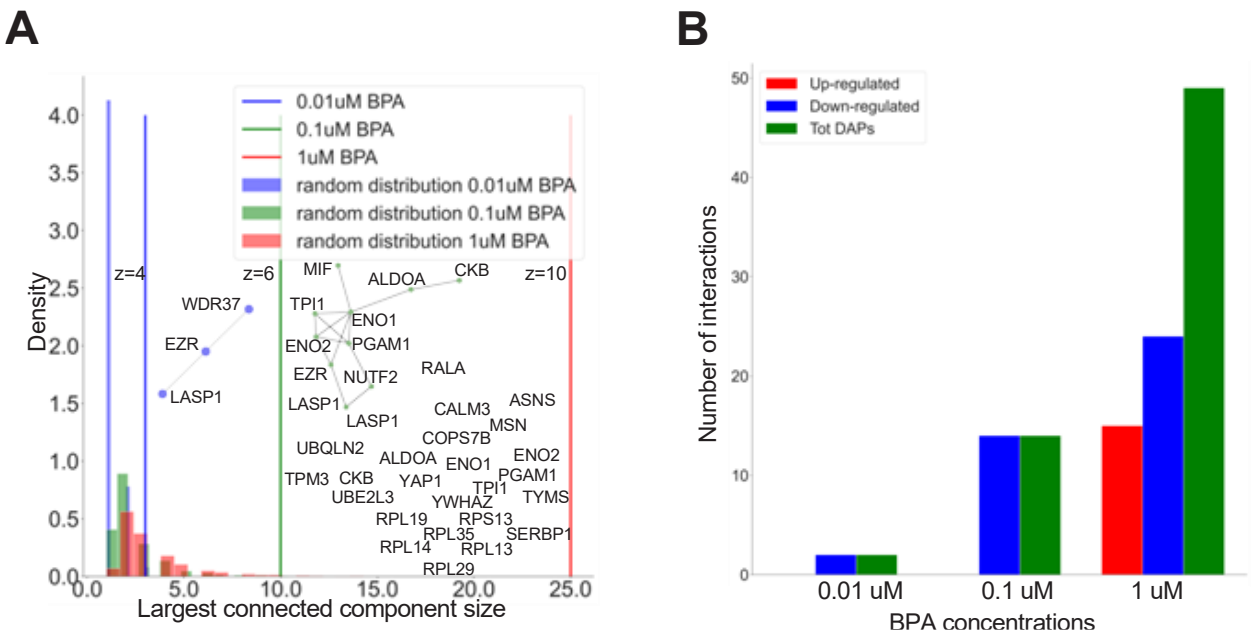

Figure 24. Distribution of the size of the largest connected component of the differentially abundant proteins in the human Protein-Protein Interactions Network at 0.01uM (blue), 0.1uM (green), 1uM (red) BPA concentrations against 10,000 random protein sets of the same size. **(B)** Bar chart representing the number of interactions among the differentially abundant proteins for each BPA-treated group, distinguishing global signature (green), up-regulated proteins (red), and down-regulated proteins (blue).

To assess the molecular interactions and the functions of the differentially abundant proteins in disease, we mapped them in a human PPI network that contained 18,816 proteins and over 470,000 physical protein interactions (Alanis-Lobato et al., 2017; Luck et al., 2020; Menche et al., 2015). In total, we observed that as BPA concentrations increased, so to the number of differentially regulated proteins and their PPI connectivity (0.01µM BPA *p*-value; 3e-5, 0.1µM BPA *p*-value 1e-09, 1µM BPA *p*-value: 2e-26, Figure 24A), which signifies an increasing quantity of proteins that contribute to the same molecular processes in a dose-dependent manner. This trend was more evident in the down-regulated proteins (Figure 24B).

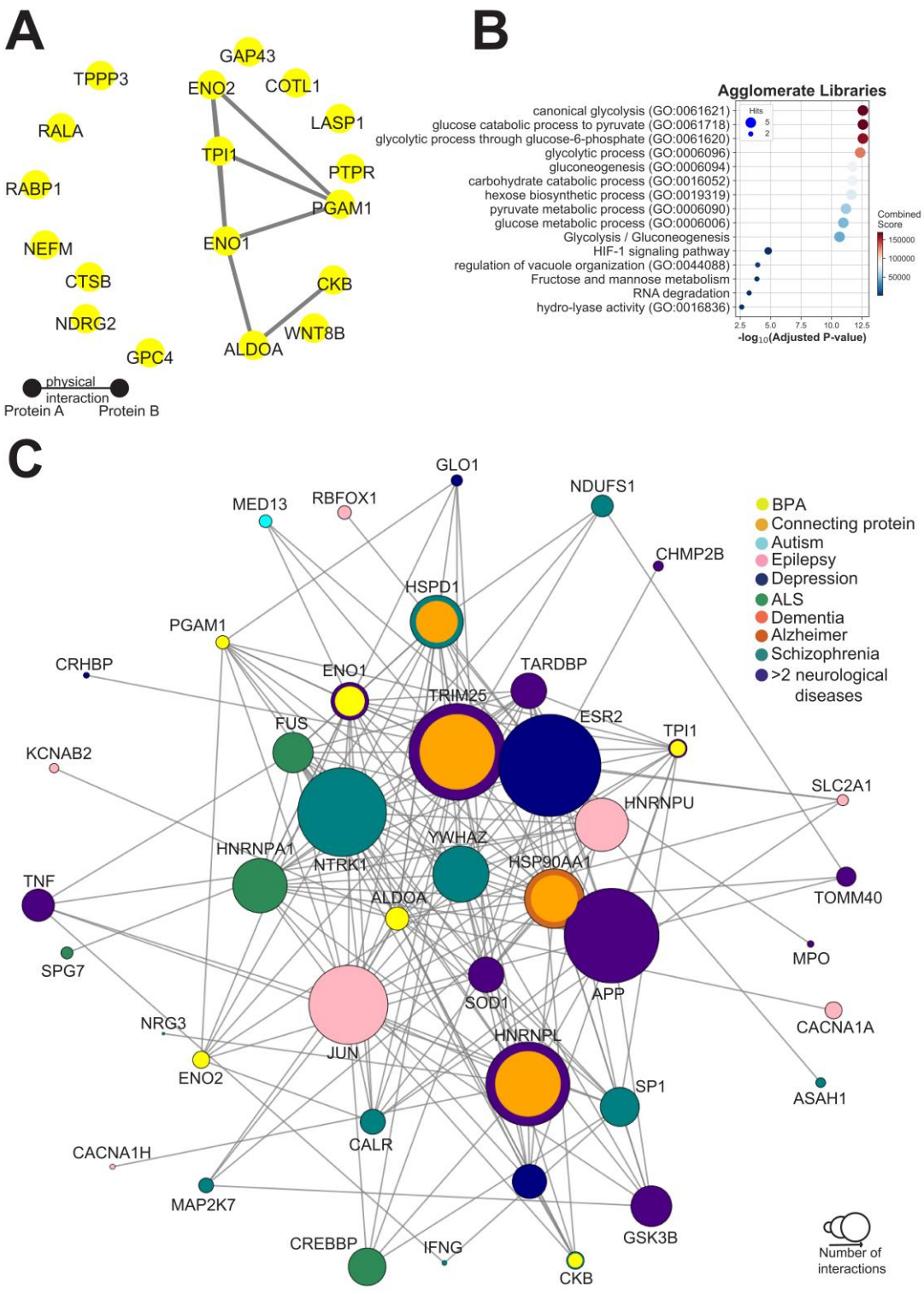

Figure 25. Network analysis of the core of BPA downregulated proteins. (A) Protein-protein interaction (PPI) network of the 18 downregulated proteins downregulated inclusively in 0.1µM and 1µM BPA-treated groups. (B) Bubble plot of the top 15 enrichment terms of six of downregulated proteins comprising the BPA downregulated core. Bubbles are plotted on their corresponding -log10 *p*-value. The size of the bubble represents the total number of proteins enriched for each term, and colour of the bubble corresponds to the combined score of the enrichment. (C) Disease-protein network with the BPA-downregulated core proteins. Each node signifies a protein, while the size of the node represents its global degree in the human PPI. The

inner-most colour of each node determines whether it is a connecting protein (orange), a disease-associated protein (each disease specified by a specific colour as shown in the figure), or one of the BPA downregulated core proteins (yellow). The colour indigo signifies a protein that is associated to >2 diseases. An outlier colour signifies a connecting protein or BPA-downregulated protein that has previously been linked to other diseases.

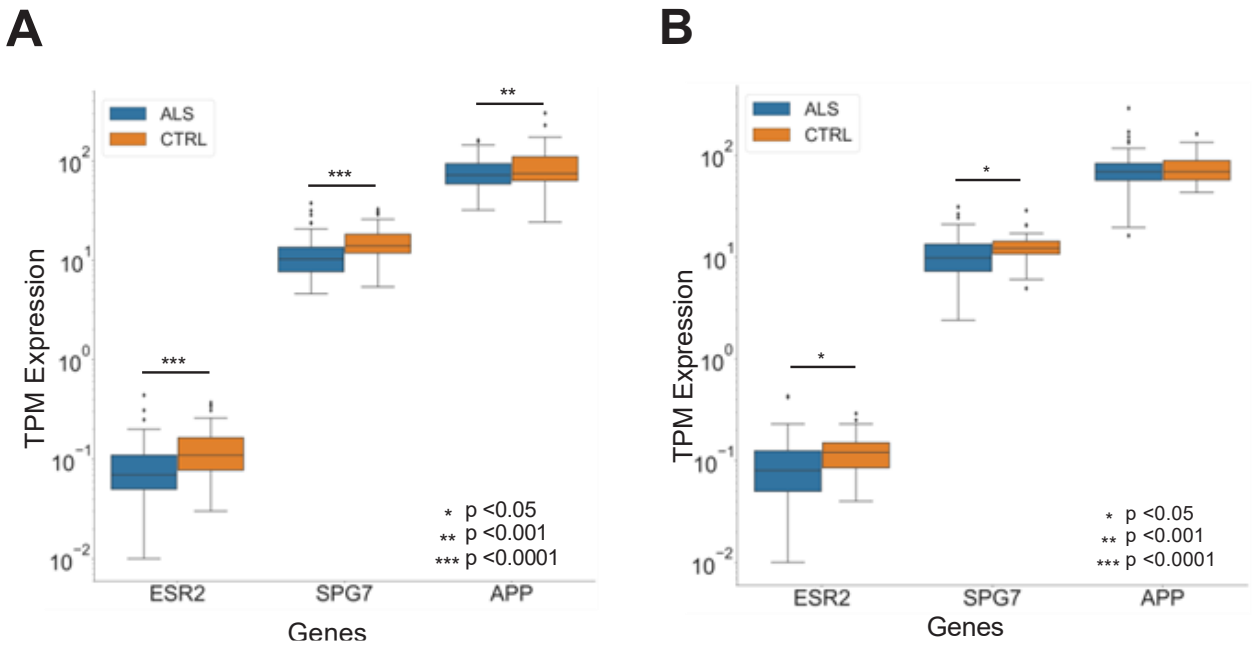

Figure 26. Boxplot of the Transcripts Per Million (TPM) expression of connecting proteins from patients affected by ALS (n=138) vs control (n=36) in the (A) cervical region and the (B) lumbar regions. Statistical significance was tested by performing a Two independent samples T-test.

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 (Figure 25A), which were enriched for both the glycolytic pathway and HIF-1 signalling pathway (Figure 25B). 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 (Piñero et al., 2015) and calculated a Jaccard index which computed a pairwise combination of their genetic overlap. We found a positive association with acute schizophrenia and enzymopathy.

To implement topological network features on the comparison between the DisGeNET disease list and the BPA downregulated core, we utilised network proximity (Guney et al., 2016), which identified many brain-related diseases on the list of predictions, for example, AD, ALS, and schizophreniform disorders.

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 (Figure 25C). 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 (McMurray et al., 2014, 2018; Tsankova et al., 2006), 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 (Figure 26A, B).

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 be underlying causes in several brain diseases.

5 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. I 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, embryos, and animal models of ART in literature.
2. In this study, I have established 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. I 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, I 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, I identified a potential pathological association between BPA perturbed proteins (ALDOA and ENO1) and several causal genes in ALS via protein interactome analysis.
5. In the present study, I observed BPA-induced Wnt alterations for the first time in a human cell-based model.
6. Finally, I demonstrated alterations to the glycolytic signalling pathway in BPA-treated human NSCs for the first time.

6 DISCUSSION

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.

Early brain developmental processes, such as neurulation, neuronal proliferation, differentiation, and migration, as well as synaptogenesis, dendritic outgrowth, and myelination, could be disrupted if the vulnerable fetal brain is exposed to environmental chemicals (Chesnut et al., 2021; Guo et al., 2013; Hornung et al., 2009; Huang et al., 2019; Kalloo et al., 2021; Ling et al., 2016; Schneider

et al., 2003). 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. Previous studies have shown that BPA exposure causes impaired proliferation of mouse NSCs (K. Kim et al., 2007), decreased proliferation of human NSCs from umbilical cord blood (Huang et al., 2019) and rodent NSCs both in vivo and in vitro (Agarwal et al., 2016; Tiwari et al., 2015). Contrastingly, in vitro studies using human and rat NSCs showed that BPA increased the proliferation of NSCs and caused differentiation impairments (Dong et al., 2021; Gill & Kumara, 2021). In addition, previous investigations have described aberrations to the differentiation of NSCs, such as precocious neurogenesis (Kinch et al., 2015), and in contrast, repressed differentiation (Agarwal et al., 2016; Dong et al., 2021; Huang et al., 2019). These opposing results could be explained by the non-monotonic property of BPA, which creates an additional challenge when attempting to reach an agreement as to the effects of BPA exposure on neuronal development, especially when considering the varied nature of experimental conditions used for investigation (Vandenberg, 2014; Villar-Pazos et al., 2017). The particular timing of developmental exposure to BPA may also impact the induced effects, and importantly, in vitro studies to date have overlooked the initial neurulation stage of brain development where NSCs first differentiate, in favour of investigations of the effects of BPA on established NSC populations.

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. In addition, we further differentiated obtained NSCs for 42 days in 2D to generate mixed cultures of neurons and glia that expressed typical neuronal and glial markers (Kobolak et al., 2020), demonstrating that our system can be used to model the effects of DOHaD-related environmental exposures from the neurulation stage of development through to neurogenesis. 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.

Spheroids provide an advantageous 3D micro-environment however it is important to acknowledge that they are constituted of one cell type only, NSCs in our study. Therefore, spheroids do not accurately replicate the complex tissue architecture present in the developing brain (Augustyniak et al., 2019). Further studies that examine BPA's effects on the development of NSCs could use brain organoids, which have the advantage of closely mimicking the intricate developmental processes of the brain. Brain organoids maintain exceptional architectural organisation and or composed of many cell types, exhibiting great promise for further toxicological applications that can support data gathered regarding BPA-induced effects on NSC spheroids (Caipa Garcia et al., 2022; Lancaster & Knoblich, 2014). 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 covered the entirety of the hiPSC to NSC differentiation (Chambers et al., 2009; Galiakberova & Dashinimaev, 2020), and for the first time, enabled the examination of the effects that BPA induces on the neural induction of hiPSCs.

Our findings showed that 14 or 21 days of BPA treatment did not affect cell viability during the neural induction. This is in support of previous in vitro studies that described no alterations to viability of NSCs and NPCs within the same concentration range, even though only acute exposure durations were studied (K. Kim et al., 2007; Tiwari et al., 2015). Additionally, we also 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.

In vivo and in vitro investigations have described BPA-induced changes to cellular ROS levels and mitochondrial dynamics in NSCs and NPCs, particularly at higher concentrations (Agarwal et al., 2016; Kim et al., 2007; Kobayashi et al., 2020; Wang et al., 2021). In this study, no significant changes to NSC mitochondrial and ROS levels were observed from qualitative analysis at day 21 of the BPA-treated neural induction in 0.01µM, 0.1µM, or 1µM BPA treated groups. Importantly, because of insufficient label penetrance and light scatter, fluorescence-based end-point imaging of

fixed NSC spheroids is mainly restricted to the outer-most layers of cells. Moreover, it is possible that any ROS alterations were compensated quickly after BPA treatments. Additionally, quantifying ROS levels in vitro is difficult, particularly when acknowledging that ROS levels are persistently altered by diffusion, compensatory mechanisms, and many chemical reactions (Murphy et al., 2022).

Acute BPA exposure at a concentration of 1μM was previously shown to reduce GFAP and MAP2 expression, markers of glial and neuronal specification, respectively, and increased the expression of Nestin and Sox2, both markers of NSC maintenance (Dong et al., 2021). Acknowledging this evidence along with previously discussed findings that BPA disturbed NSC neurogenesis/differentiation, we examined if BPA affected the rate of differentiation of hiPSCs to NSCs. We did not observe any alterations to the protein or mRNA levels of neuroectodermal markers Sox1 and Nestin. Moreover, we observed no changes to the mRNA levels of Tub3, an immature neuronal marker that could signify neuronal differentiation (Katsetos et al., 2003), or Sox2, a multipotency marker in non-committed NSCs (Shimozaki, 2014), indicating that there was no change to the rate of the differentiation.

Much evidence demonstrates BPA-induced alterations to key molecular pathways that play critical roles in NSC maintenance and differentiation. It has been reported that human NSCs exposed to 1μM BPA showed alterations to pathways that are important for maintaining NSC stemness, including the p53, TGF-β and ERRα signalling pathways (Dong et al., 2021). Moreover, both in vitro and in vivo rat studies showed that BPA interrupts the canonical Wnt signalling pathway, which maintains a crucial role in the proliferation and differentiation of NSCs and NPCs, in addition to the pathophysiology of several NDDs (Fang et al., 2015; Mulligan & Cheyette, 2016; Tiwari et al., 2015, 2016; Üstündağ & Emekli-Alturfan, 2020). However, despite these findings, there remains a continued debate regarding the molecular implications and effects of BPA exposure during the embryonic and fetal stages of brain development, as well as its associations to brain disorders.

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). Also, a lack of GAP43 expression causes errors to the neuronal and glial fate commitment and differentiation of multipotent neural progenitors (Mani et al., 2001; Shen et al., 2004, 2008). The proteomics analysis also detected decreases to TPPP3 (p20) inclusively in all BPA-treated groups. p20 is a member of the TPPP family of proteins and is a cyclin-dependent kinase inhibitor with a prominent role in proliferation during most developmental stages (Oláh et al., 2017; Shukla et al., 2018). 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. Intriguingly, it has been previously demonstrated that the capacity of GPC4 to regulate stem cell fate depends on the positive modulation of the canonical Wnt signalling pathway (Fico et al., 2012). 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). Contrarily, we saw an increase to FABP7 protein levels in all groups treated with BPA. FABP7 is typically expressed in NSCs and NPCs (Knobloch, 2017). The expression of FABP7 has been shown to peak during NSC to radial glial progenitor transition and plays a part in maintaining glial progenitors (Arai et al., 2005; Matsumata et al., 2016). Thus, the BPA-induced increases to FABP7 could also suggest that BPA-treated NSCs are at a more ‘primed’ phase for transition towards radial glial progenitors.

Taken together, we discovered BPA-induced alterations to the abundance of many proteins with critical functional roles in the maintenance of NSCs; complementing current literature and providing an enhanced understanding of the potential molecular mechanisms responsible for proliferation and differentiation changes in NSCs derived from the BPA treated neural induction. The changes to the protein levels observed in the present study may suggest that NSCs exposed to BPA over the course of the neural induction are at an advanced phase of differentiation, more closely akin to NPCs, which exhibit a diminishing proliferative potential compared to NSCs. Therefore, an interesting avenue for further research could be to determine if BPA exposure prematurely coaxes NSCs to an advanced 'primed' NPC state, which would provide an explanation for the suppressed proliferation and precocious reduction of the NSC pool. A current limitation concerning the use of MS-based proteomics is the under-sampling or un-detection of peptides that are low in abundance, because of dynamic range limitations. Proteins of relatively low abundance, including transcription factors, are therefore commonly underrepresented in proteomic studies, and therefore it remains possible that there were several other important proteome changes caused by BPA exposure that were not identified in this study. Current challenges regarding the measurement of peptides of low abundance may be addressed in the future as the sensitivity of mass spectrometers is improved, which could facilitate a more exhaustive examination of proteome-wide changes in NSCs exposed to BPA (Ding et al., 2013; Fonslow et al., 2011; Timp & Timp, 2020).

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.

Findings from previous reports as well as this study could explain the mechanisms induced by BPA that led to the aberrant expression of glycolytic proteins. Estrogen receptors (ERs) are ligand-

activated and ubiquitous transcription factors and BPA is known to have a strong binding affinity to ERs. Estrogen-dependent signalling pathways, with roles in modulating cellular proliferation and differentiation in a range of cell types and tissues, including NSCs during brain development, are known to be regulated by ERs (Bustamante-Barrientos et al., 2021). Of interest, ERs utilise intracellular effects via PI3K/ATK signalling, a pathway known for the promotion of growth, metabolism, and proliferation in reaction to extracellular signals (Hoxhaj & Manning, 2020; MacKay & Abizaid, 2018). PI3K-Akt signalling processes are also known to maintain important functions in promoting glycolysis (Xie et al., 2019). A downstream effector of the PI3K-Akt pathway is HIF-1α, a protein of the HIF-1 signalling pathway. HIF-1α is commonly known to be regulated in an oxygen-dependent manner by prolyl hydroxylases, however, in non-hypoxic conditions its regulation is also under the control of the PI3K/Akt signalling pathway (Masoud & Li, 2015). The HIF-1 signalling pathway has also been reported to modulate metabolic transporters and enzymes that promote glycolysis (Del Rey et al., 2017; Kim et al., 2014). In the present study we showed BPA-induced decreases to proteins of the HIF-1 signalling cascade in both 0.1μM and 1μM treated groups. Furthermore, another effector protein of the PI3K/Akt signalling pathway is p53, which plays a role in the suppression of glycolysis (Kim et al., 2014). P53 signalling alterations were previously described in human NSCs upon exposure with 1μM BPA (Dong et al., 2021). Thus, a promising direction for further study could be the examination of the effects of BPA on the metabolic condition of NSCs and neurons. Such investigations have the potential to elucidate the mechanisms underlying the altered proliferation and differentiation of NSCs and neurons due to BPA-induced errors to the glycolytic pathway, which is yet to be investigated in literature.

Several studies have highlighted the relationship between NDDs, NDs and glycolysis in the brain (Li & Sheng, 2022; Nascimento & Martins-de-Souza, 2015; Zhang et al., 2021). NSC fate regulation is partly regulated via metabolic gradients (Angelopoulos et al., 2022), and aberrant regulation of the glycolytic metabolism during pregnancy has been linked to neural tube defects (Keuls et al., 2020; Paddock et al., 2017). Moreover, reduced glucose and oxygenation rates are associated to NDs, ageing (Camandola & Mattson, 2017), and restricted synaptogenesis during embryonic brain development (Goyal et al., 2014).

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 summary, we show that BPA exposure could result in important metabolic alterations during brain development, with the potential to deter several crucial processes in development such as neural tube formation and synaptogenesis and increasing the risk for the development of several brain disorders throughout life.

In conclusion, we present a novel NAM for investigating the effects of environmental chemical exposures 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 periconceptional 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.

7 SUMMARY (EN)

Increasing evidence in support of the DOHaD hypothesis emphasises the critical requirement for improved models that can generate translatable, human-relevant data. Such models can enhance the current understanding of the effects of DOHaD-related alterations, including the effects of environmental chemical exposures on the embryonic brain, and ART-associated IDs that affect brain development. BPA exposure has been linked to a range of brain diseases and aberrant neurodevelopment. It has been shown that BPA induces disturbances to vital NSC characteristics, including both proliferation and differentiation, yet the molecular alterations responsible for these changes remain under investigation. In the present study, we firstly critically assessed current literature and showed that patterns of vulnerable imprints in reprogrammed iPSCs can be used to improve our understanding of the association between ART and several brain-related IDs. Additionally, using a detailed characterization, we demonstrated that our model for the neuronal differentiation of hiPSCs is suitable for toxicological applications and assessing the effects of environmental chemicals on embryonic brain development from neurulation through to neurogenesis. We then examined the effects of a repeated-dose, environmentally realistic exposure of BPA during the neural induction of hiPSCs to NSCs in 3D format, effectively modelling a real-life exposure scenario during the neurulation stages of brain development. By analyzing spheroid morphology, we showed that BPA exposure at concentrations of 0.01µM, 0.1µM and 1µM BPA reduced the average size of spheroids by day 21 of the differentiation. Quantitative proteomics analysis demonstrated many differentially expressed proteins in all BPA-treated groups with critical roles in the maintenance and proliferation of NSCs (e.g., Wnt-8B, GPC4, p20, GAP43, FABP7). Moreover, a network analysis showed BPA-induced changes to the glycolytic pathway; potentially linking BPA exposure to glycolysis aberrations which could ultimately contribute to inhibited NSC proliferation, as well as the pathophysiology of several NDs and NDDs such as ASD, ALS, and intellectual disability. The present study improves the knowledge of BPA's effects on NSCs, which was previously and primarily based on acute, frequently high-dose exposures from human genome-wide association studies as well as in vivo and in vitro rodent models. Our novel, 3D human cell-based model of the neural induction facilitated a real-life relevant and repeated exposure to low-doses of BPA, providing an enhanced mechanistic understanding into the consequences of BPA exposure on human embryonic brain development, and therefore, neurological disorders later in life. Our model of the differentiation of hiPSCs to mixed cultures of neurons and glia can be used for further in vitro testing of the effects of DOHaD-related exposures on embryonic brain development, including disease modelling for ART-associated IDs, and for the assessment of the effects of chronic environmental chemical exposure.

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9 PUBLICATION LIST

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.
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International poster presentations:

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- **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.
- **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.

Conference oral presentations:

- **Horánszky A**, Zana M, Dinnyés A. The impact of environmental exposures on the neuronal differentiation of pluripotent stem cells. **HUNDOC** 2022, Budapest, Hungary. January 2022.
- **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.

10 APPENDICES

Appendix A: Supplementary Table 1. Antibodies used in this work for immunocytochemical analysis.

Manufacturer/ Cat. No.	Host/ Clonality	Target	Isotype	Dilution
Sigma-Aldrich/ MAB5326	Mouse/ Monoclonal	Nestin	IgG	1:200
R&D systems/ AF3369	Goat/ Polyclonal	Sox1	IgG	1:100
Sant Cruz/ sc365823	Mouse/ Monoclonal	Sox2	IgG1	1:100
Cell Signal/ 5741s	Rabbit/ Monoclonal	Vimentin	IgG	1:100
Abcam/Ab5392	Chicken	MAP2	IgG	1:2500
Abcam/Ab78078	Mouse	TUBB3	IgG	1:1000
ThermoFisher/ MA5-12023	Mouse	GFAP	IgG	1:500
Abcam/Ab16667	Rabbit	Ki67	IgG	1:250
Abcam/Ab8135	Donkey	Nf200	IgG	1:1000
Abcam/Ab3209	Goat	Oct4	IgG	1:500
SantaCruz/sc-33760	Rabbit	Nanog	IgG	1:1000
SantaCruz/sc-21706	Mouse	TRA-1-81	IgM	1:500

Supplementary Table 2. Primers sequences used for RT-qPCR analysis.

Gene	Forward sequence	Reverse sequence
Vimentin	GACCAGCTAACCAACGACAAAG	CGCATTGTCAACATCCTGTCTG
Sox1	TAGTAAGGCAGGTCCAAGCA	GGGTGGTGGTGGTAATCTCT
Sox2	ACCAGCTCGCAGACCTACA	TCGGACTTGACCACCGAAC
Nestin	ACTGAAGTCTGCGGGACAAG	CAGTGGTGCTTGAGTTTCTG
OCT4	AAAGCGAACCAGTATCGAGAAC	GCCGGTTACAGAACCACACT
Pax6	GCCAGCAACACACCTAGTCA	TGTGAGGGCTGTGTCTGTTC
Tub3	AACGAGGCCTCTTCTCACAA	GGCCTGAAGAGATGTCCAAA
Map2	TTGTCTCTAACCGAGGAAGCA	TCGTTGTGTCGTGTTCTCAA
BMI1	ACAAAGAGAAATCTAAGGAGGAGG	CCAGGTATAAATGTAGGCAATATCC
DCX	TATGCGCCGAAGCAAGTCTC	TACAGGTCCTTGTGCTTCCG
Hes1	GCACAGAAAGTCATCAAAGCCT	CATTTCCAGAATGTCCGCCTTC
CDK4	CTTCTGCAGTCCACATATGCAACA	CAACTGGTCGGCTTCAGAGTTTC
Dmrta1	CTTGAGACAGGCCAGTGGTT	TTTGTTTCCCAATGACACCA
TGFB1	CAAGCAGAGTACACACAGCATA	GTATCGCCAGGAATTGTTGCT

Supplementary Table 3A. Summary of differentially abundant proteins in the 0.01µM BPA treated group at day 21 of the BPA-treated neural induction.

Genes	log2 fold change (D21L vs D21)	P-value adjusted (BH)	Differentially abundant
FABP7	0.42	0.046178691	upregulated_in_brain_D21_l
EZR	-0.44	0.000395435	downregulated_in_brain_D21_l
LASP1	-0.45	0.025279011	downregulated_in_brain_D21_l
GPC4	-0.66	0.016437096	downregulated_in_brain_D21_l
CRIP2	-0.76	0.04241434	downregulated_in_brain_D21_l
CRABP1	-0.84	1.11E-11	downregulated_in_brain_D21_l
TPPP3	-0.85	0.016834248	downregulated_in_brain_D21_l
WNT8B	-0.87	0.000160699	downregulated_in_brain_D21_l
GAP43	-1.02	0.000424919	downregulated_in_brain_D21_l
NR2F1	-1.40	0.000268619	downregulated_in_brain_D21_l
WLS	-1.55	0.011711296	downregulated_in_brain_D21_l
WDR37	-1.88	0.004688027	downregulated_in_brain_D21_l

Supplementary Table 3B. Summary of differentially abundant proteins in the 0.1µM BPA treated group at day 21 of the BPA-treated neural induction.

Genes	log2 fold change (D2m vs D21)	P-value adjusted (BH)	Differentially abundant
FABP7	0.52	0.000105928	upregulated_in_brain_D21_m
EZR	-0.44	7.64E-07	downregulated_in_brain_D21_m
LASP1	-0.41	0.000693236	downregulated_in_brain_D21_m
GPC4	-0.72	0.001878068	downregulated_in_brain_D21_m
CRIP2	-	-	-
CRABP1	-0.89	0	downregulated_in_brain_D21_m
TPPP3	-0.90	0.000432738	downregulated_in_brain_D21_m
WNT8B	-0.62	0.001352125	downregulated_in_brain_D21_m
GAP43	-0.62	0.00056688	downregulated_in_brain_D21_m
NR2F1	-	-	-
WLS	-0.92	0.017249463	downregulated_in_brain_D21_m
WDR37	-	-	-
KIF11	1.15	0.000439073	upregulated_in_brain_D21_m
BANF1	0.97	5.98E-07	upregulated_in_brain_D21_m
PRSS23	0.71	0.012783087	upregulated_in_brain_D21_m
SFRP2	0.64	0.000225423	upregulated_in_brain_D21_m
TF	0.52	2.34E-09	upregulated_in_brain_D21_m
LIN28A	0.45	0.001423469	upregulated_in_brain_D21_m
SMOC1	0.44	0.048498866	upregulated_in_brain_D21_m
SALL2	0.42	0.005950051	upregulated_in_brain_D21_m
ASNS	0.42	0.022710162	upregulated_in_brain_D21_m
VAT1L	-0.38	0.031404896	downregulated_in_brain_D21_m

COTL1	-0.38	0.000302219	downregulated_in_brain_D21_m
RALA	-0.39	0.042929709	downregulated_in_brain_D21_m
NUTF2	-0.40	0.01107247	downregulated_in_brain_D21_m
PGAM1	-0.40	9.86E-05	downregulated_in_brain_D21_m
GAA	-0.40	0.006604962	downregulated_in_brain_D21_m
LLGL1	-0.40	4.17E-06	downregulated_in_brain_D21_m
ENO2	-0.43	0.000193486	downregulated_in_brain_D21_m
CKB	-0.43	3.86E-09	downregulated_in_brain_D21_m
ENO1	-0.46	0	downregulated_in_brain_D21_m
STMN1	-0.47	0.000672953	downregulated_in_brain_D21_m
ALDOA	-0.47	3.39E-09	downregulated_in_brain_D21_m
CTSB	-0.48	0.006140732	downregulated_in_brain_D21_m
TPI1	-0.51	0	downregulated_in_brain_D21_m
PTPRF	-0.54	0.000155232	downregulated_in_brain_D21_m
MIF	-0.55	0.01963405	downregulated_in_brain_D21_m
TPP1	-0.62	0.002037701	downregulated_in_brain_D21_m
SNRPD1	-0.67	0.017455364	downregulated_in_brain_D21_m
UFD1L	-0.67	0.006854611	downregulated_in_brain_D21_m
NEFM	-0.70	3.73E-07	downregulated_in_brain_D21_m
NDRG2	-0.82	0.002037701	downregulated_in_brain_D21_m

Supplementary Table 3C. Summary of differentially abundant proteins in the 1µM BPA treated group at day 21 of the BPA-treated neural induction.

Genes	log2 fold change (D21h vs D21)	P-value adjusted (BH)	Differentially abundant
FABP7	0.47	0.001555063	upregulated_in_brain_D21_h
LASP1	-0.39	0.000980171	downregulated_in_brain_D21_h
GPC4	-0.77	0.000245633	downregulated_in_brain_D21_h
CRABP1	-0.81	0	downregulated_in_brain_D21_h
TPPP3	-1.05	0.001199266	downregulated_in_brain_D21_h
WNT8B	-0.50	0.022451677	downregulated_in_brain_D21_h
GAP43	-0.73	0.001652906	downregulated_in_brain_D21_h
BANF1	0.96	0.000129	upregulated_in_brain_D21_h
PRSS23	0.67	0.018722119	upregulated_in_brain_D21_h
SFRP2	0.79	1.28E-06	upregulated_in_brain_D21_h
SALL2	0.48	0.00334	upregulated_in_brain_D21_h
ASNS	0.42	0.0463	upregulated_in_brain_D21_h
COTL1	-0.45	5.00E-05	downregulated_in_brain_D21_h
RALA	-0.50	0.005741194	downregulated_in_brain_D21_h
PGAM1	-0.41	6.76E-06	downregulated_in_brain_D21_h
ENO2	-0.49	6.19E-05	downregulated_in_brain_D21_h
CKB	-0.41	1.95E-09	downregulated_in_brain_D21_h
ENO1	-0.48	0	downregulated_in_brain_D21_h
ALDOA	-0.46	1.32E-09	downregulated_in_brain_D21_h
CTSB	-0.61	0.009871541	downregulated_in_brain_D21_h
TPI1	-0.43	1.37E-10	downregulated_in_brain_D21_h

PTPRF	-0.68	0.01948466	downregulated_in_brain_D21_h
NEFM	-0.42	0.005741194	downregulated_in_brain_D21_h
NDRG2	-0.76	0.003994365	downregulated_in_brain_D21_h
HIST1H1C	1.26	0.000237836	upregulated_in_brain_D21_h
RPS10;RPS10P5	1.20	0.00238	upregulated_in_brain_D21_h
HIST1H1E	1.12	2.58E-12	upregulated_in_brain_D21_h
INS;INS-IGF2	1.01	0.006746338	upregulated_in_brain_D21_h
HIST1H1B	0.95	0.000667932	upregulated_in_brain_D21_h
RPL35	0.69	4.72E-05	upregulated_in_brain_D21_h
RPL29	0.67	0.001265098	upregulated_in_brain_D21_h
H1FX	0.60	0.026587644	upregulated_in_brain_D21_h
RPL13	0.58	1.49E-05	upregulated_in_brain_D21_h
RPL14	0.54	0.045492741	upregulated_in_brain_D21_h
HIST1H3A	0.49	0.000132136	upregulated_in_brain_D21_h
RPL19	0.46	0.00301	upregulated_in_brain_D21_h
RPS13	0.45	0.002151535	upregulated_in_brain_D21_h
ATP5H	0.44	0.000466622	upregulated_in_brain_D21_h
HIST1H4A	0.39	8.53E-05	upregulated_in_brain_D21_h
TYMS	0.39	0.039660165	upregulated_in_brain_D21_h
HIST2H2AA3	0.38	8.59E-05	upregulated_in_brain_D21_h
ARMCX3	0.38	0.00971	upregulated_in_brain_D21_h
YWHAZ	-0.38	2.57E-06	downregulated_in_brain_D21_h
MSN	-0.38	2.87E-07	downregulated_in_brain_D21_h
CALM3	-0.40	2.01E-05	downregulated_in_brain_D21_h
PLIN3	-0.41	0.003207002	downregulated_in_brain_D21_h
HEXB	-0.41	0.00015918	downregulated_in_brain_D21_h
PSMB1	-0.42	0.003164589	downregulated_in_brain_D21_h
TPM3	-0.42	0.00438	downregulated_in_brain_D21_h
COPS7B	-0.46	0.046509284	downregulated_in_brain_D21_h
ASPH	-0.47	0.010193605	downregulated_in_brain_D21_h
GGCT	-0.47	0.038698153	downregulated_in_brain_D21_h
YAP1	-0.47	0.000466622	downregulated_in_brain_D21_h
ARL6IP5	-0.47	0.047108894	downregulated_in_brain_D21_h
SERBP1	-0.53	2.89E-07	downregulated_in_brain_D21_h
HMGN3	-0.54	0.035336211	downregulated_in_brain_D21_h
LAMP2	-0.60	0.025293402	downregulated_in_brain_D21_h
GM2A	-0.62	0.01948466	downregulated_in_brain_D21_h
HSBP1	-0.62	0.039660165	downregulated_in_brain_D21_h
UBE2L3	-0.67	8.53E-05	downregulated_in_brain_D21_h
ARSB	-0.71	0.042615791	downregulated_in_brain_D21_h
GPM6A	-0.77	0.014003349	downregulated_in_brain_D21_h
NIF3L1	-0.86	0.00264026	downregulated_in_brain_D21_h
UBQLN2	-0.87	0.002604904	downregulated_in_brain_D21_h
NDUFAB1	-1.23	0.0107775	downregulated_in_brain_D21_h
SLC25A4	-1.42	3.00E-06	downregulated_in_brain_D21_h

Appendix B:

Compositions of culture media used in this study:

1. **Thawing medium**

Reagent	Final concentration
Complete NMM media	100%
ROCK inhibitor (10 mM)	10 µM

2. **Freezing medium**

Reagent	Final concentration
FBS	90%
DMSO	10%

3. **Neural Induction medium**

Reagent	Final concentration
DMEM/F12	50%
Neurobasal medium	50%
Non-essential amino acids	1%
B-27 supplement minus vitamin A) (50X)	2%
N-2 supplement (100X)	1%
L-Glutamine (200 mM)	1%
Pen/Strep (10 000 U/ml)	1%
bFGF (100 µg/ml)	10 ng/ml
β-Mercaptoethanol	100 µM
LDN-193189 HCL	200 nM
SB431542 Hydrate	10 µM
Insulin	5 µg/ml

4. **NPC maintenance medium**

Reagent	Final concentration
DMEM/F12	50%

Neurobasal medium	50%
Non-essential amino acids	1%
B-27 supplement (50X)	2%
N-2 supplement (100X)	1%
L-Glutamine (200 mM)	1%
Pen/Strep (10 000 U/ml)	1%
EGF (100 µg/ml)	10 ng/ml
bFGF (100 µg/ml)	10 ng/ml

5. Neuronal maintenance medium (NMM)

Reagent	Final concentration
DMEM/F12	50%
Neurobasal medium	50%
Non-essential amino acids	1%
B-27 supplement (50X)	2%
N-2 supplement (100X)	1%
L-Glutamine (200 mM)	1%
Pen/Strep (10 000 U/ml)	1%

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