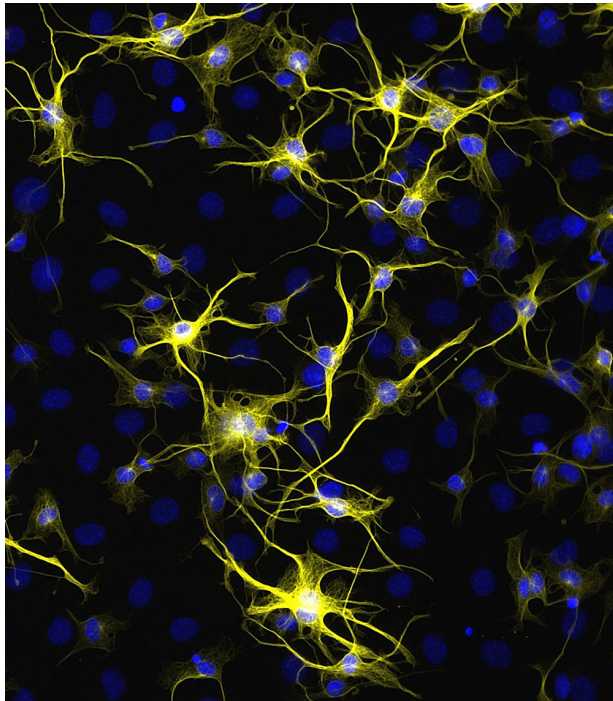




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# Optimization of an *In-vitro* System for Testing Developmental Neurotoxicity Induced by Oestrogen, Androgen and Thyroid Disruption



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## ABSTRACT

In recent times, endocrine disrupting chemicals (EDCs) have been associated with the rise in neurodevelopmental disorders such as autism, attention deficit hyperactivity disorder (ADHD) and decreased intelligence quotient (IQ) in children. This effect is suspected to be induced at pre-/peri-natal development, via an alteration in hormonal signaling, thus interfering with neuronal differentiation, with subsequent effect on normal brain development and function in exposed children. This issue increases the need for chemical screening for potential developmental neurotoxicity effect (DNT). The current available EDC induced DNT test guideline is based on *in-vivo* testing that requires animal use. Here, a multipotent neural progenitor cell line, the C17.2 cell-line, generated from neural stem cells of the external germinal layer of mouse cerebellum, with potential to differentiate to neurons or astrocytes, is introduced for *in-vitro* EDC induced DNT testing. This project focused on optimizing the C17.2 cell-line for the detection of EDC-induced DNT with emphasis on the disruption of the oestrogen, androgen, and thyroid hormone systems. It aimed at validating the involvement of oestrogen, androgen, and thyroid hormone on molecular and cellular endpoints relevant for the differentiation of the C17.2 cells. Herein, the cells were exposed to the hormonal agonist and antagonist at a range of concentrations for a 10-day differentiation period. After exposure, LDH, viability assay and morphological changes (percentage of neurons in culture and neurite outgrowth) were evaluated. The results showed no morphological changes induced by androgen receptor (AR) agonist/antagonist at relevant physiological concentrations. The thyroid receptor (TR) agonist and antagonist on the other hand showed a response in the form of increased neurite outgrowth in relation to the negative control at a concentration range of 40-200 nM and 40 nM respectively. The oestrogen receptor (ER) antagonist at 100 nM also increased percentage neuron in culture. Additionally, *in-silico* analysis of microarray and RNA sequencing data were used to map out target genes regulated by ER, AR and TR and involved in neurodevelopment. With this approach, 29 marker genes were identified. Validation of the marker genes by means of gene expression (qPCR) was carried out, ER and TR agonist/antagonist were observed to modulate the expression of examined genes. In summary, the model could not be established for detecting EDC induced DNT via androgenic and oestrogenic pathway, while it is a promising model for identifying DNT induced by thyroid hormone signalling disruption.

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## ABBREVIATIONS

ADHD	Attention deficit hyperactivity disorder
AR	Androgen receptor
ASDs	Autism spectrum disorders
BDNF	Brain-derived neurotrophic factor neural
BMP4	Bone morphogenetic protein 4
BPA	Bisphenol A
BSA	Bovine serum albumin
C3	Complement C3
CCN2	Cellular communication network factor 2
CDH13	Cadherin 13
CDH6	Cadherin 6
CLU	Clusterin
CNS	Central nervous system
CX3CL1	C-X3-C motif chemokine ligand 1
DDT	Dichlorodiphenyltrichloroethane
DLL1	Delta like canonical Notch ligand 1
DMEM	Dulbecco's modified Eagle's medium
DMEM/F-12	Dulbecco's modified Eagle's medium: Nutrient mixture Ham's F12 (1:1)
DMSO	Dimethyl Sulfoxide
DNT	Developmental neurotoxicity
ED	Endocrine disruption
EDCs	Endocrine disrupting chemicals
ER	Oestrogen receptor
ER- $\alpha$	Oestrogen receptor alpha
ER- $\beta$	Oestrogen receptor beta
FBS	Fetal bovine serum
FOS	Fos proto-oncogene, AP-1 transcription factor subunit
GFAP	Glial fibrillary acidic protein
GPR30	G protein-coupled receptor 30
HDAC11	Histone deacetylase 11
HEY1	Hes related family bHLH transcription factor with YRPW motif 1
HS	Horse serum
ID	Intellectual disability
KCNA1	Potassium voltage-gated channel subfamily A member 1
LOX	Lysyl oxidase
MAPT	Microtubule associated protein tau
MFSD2A	Major facilitator superfamily domain containing 2A
MICOS10-	
MME	Membrane metalloendopeptidase
MYOC	Myocilin
NBL1/NBL1	DAN family BMP antagonist
ND	Neurodevelopmental
NFs	Neurotrophic factors
NGF	Neural growth factor
NOS1	Nitric oxide synthase 1

PBS	Phosphate-buffered saline
PCB	Polychlorinated biphenyl
PDGFRA	Platelet derived growth factor receptor alpha
PFA	Paraformaldehyde
PRDM8	PR/SET domain 8
PTGS2	Prostaglandin-endoperoxide synthase 2
RORC	RAR related orphan receptor C
S100B	S100 calcium binding protein B
S1PR1	Sphingosine-1-phosphate receptor 1
SELENOP	Selenoprotein P
SPRY4	Sprouty RTK signaling antagonist 4
SULF1	Sulfatase 1
T3	3,5,3'-Triiodothyronine
T4	Thyroxine
THBS3	Thrombospondin 3
THRA	Thyroid Hormone Receptor Alpha
THRB	Thyroid Hormone Receptor Beta
THs	Thyroid hormones
TMOD1	Tropomodulin 1
TNC	Tenascin C
TR	Thyroid receptor
TR $\alpha$ 1	Thyroid Hormone Receptor Alpha 1
TR $\alpha$ 2	Thyroid Hormone Receptor Alpha 2
TR $\beta$ 1	Thyroid Hormone Receptor Beta 1
TR $\beta$ 2	Thyroid Hormone Receptor Beta 2
VSNL1	Visinin like 1

## 1.0 INTRODUCTION

### 1.1 Endocrine disrupting chemicals (EDCs)

The endocrine system regulates critical biological functions such as metabolism, development, reproduction, and behaviour in animals (Street et al., 2018). Xenobiotics, released into the environment as a product of human activity, can potentially alter the endocrine system of wildlife and humans (Street et al., 2018). These chemicals with the potential to produce detrimental effects to the endocrine system are known as Endocrine Disrupting Chemicals (EDCs). According to Solecki (2017) from WHO/IPCS, (2002) “An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations”. Among the over 86,000 commercially available chemicals listed by Toxic Substances Control Act (TSCA) (EPA, 2019), about 1000 are acknowledged as potential EDCs. Some examples are phthalates, bisphenol A (BPA), flame retardants, alkylphenols, dioxins, polycyclic aromatic hydrocarbons, pesticides, and some metals (Street et al., 2018). EDCs can act, for example, by interfering with hormonal transport, activation, or co-activators that are important for hormone receptor signalling (Street et al., 2018; Thomas Zoeller et al., 2012).

The best studied targets of EDCs are hormone receptors belonging to the family of nuclear receptors. Nuclear receptors are ligand-regulated transcription factors with roles in organism development, reproduction, homeostasis, and metabolism (Laudet and Gronemeyer, 2002; Sakkiiah et al., 2018). All nuclear receptors have three major functional domains; (1) the N-terminal domain (NTD), (2) the DNA-binding domain (DBD), and the (3) ligand-binding domain (LBD). They have similar ligand-binding domains with 12 helices. The helix 3 (H3), H7 and H10 form the active site also known as hydrophobic pocket (Sakkiiah et al., 2018). However, the active site differs among the receptors (Sakkiiah et al., 2018; Wurtz et al., 1996). H12 functions as a lid at the entrance of the ligand binding pocket (Sakkiiah et al., 2016). Upon binding of ligand to the active site, the LBD undergoes a conformational change in the activation function 2 site (AF2) (Sakkiiah et al., 2018). This leads to the dissociation of chaperones and/or co-repressors and dimerization depending on the NR, to stimulate DNA binding for gene expression regulation, via the assembly and interaction with cofactors (coactivators or corepressors) (Ambrosino et al., 2010). The ligand-receptor complexes bind to response elements at the promoter regions of the target genes to control either activation or repression of gene expression (Huang et al., 2004; Lee et al., 2013).

EDCs that directly bind to hormone receptors most often have a phenolic structure that enables them to act as endogenous hormones (Lee et al., 2013). As a result of this, they can act as agonist (activate) or antagonist (block) on a hormone receptor. An agonist promotes receptor transcriptional activity whereas receptor antagonists lead to the recruitment of co-repressors such as NcoR and SMRT, thus making it less likely to activate transcription (Hodgson et al., 2008; Luccio-Camelo & Prins, 2011). As such, the DNA-bound receptor complex may exert positive or negative effects on expression of the downstream target genes, respectively, depending on the cell and promoter context (Luccio-Camelo & Prins, 2011).

## **1.2 Hormonal regulation of brain development**

### **1.2.1 The oestrogenic pathway**

Oestrogen plays an important role in reproductive and non-reproductive tissues such as the brain, lungs, colon, prostate, and cardiovascular system (Shanle & Xu, 2011). Oestrogenic activity in the brain regulates the ovarian cycle through the hypothalamus-pituitary-gonadal axis, sexual differentiation of the brain and the development and function of neurons and glia (Zsarnovszky et al., 2005). Oestrogen exerts its cellular action mainly through intracellular oestrogen receptors (ERs), which are NRs and thus act as transcription factors to regulate oestrogen-responsive gene expression (Lee et al., 2013; Zsarnovszky et al., 2005). Yet, there is also a membrane-bound ER (GPR30). Nuclear ERs mediate transcriptional activation (genomic pathway) within hours, while the membrane ER activate ligand-independent pathways (non-genomic pathways) within minutes of exposure to ligands via kinase signalling cascades (Jepsen et al., 2000; Lee et al., 2013; Suzuki et al., 2008).

The oestrogen nuclear receptor occurs in two isomeric forms, ER- $\alpha$  and ER- $\beta$ . Both forms are expressed in the brain, lung, uterus, breast, heart, and intestine (Shanle & Xu, 2011; Taylor & Al-Azzawi, 2000). In mouse brain, ER $\alpha$  shows unique expression in tissues like the pituitary gland, cerebral cortex, amygdala, hypothalamus, basal ganglia, while ER $\beta$  is expressed in the cerebellum, cerebral cortex, amygdala, thalamus, hypothalamus, midbrain, substantia nigra, amygdala, olfactory region, and pituitary gland (The human protein atlas, 2020; Mitra et al., 2003). Both isomers have distinct molecular mechanisms and distribution on oestrogen-dependent specific tissue, and their transcriptional activation differs depending on the promoter, ligand affinity and cell type (Samudio et al., 2001). For example, Cyclin D1 levels are increased by ER-  $\alpha$  and decreased by ER-  $\beta$  (Lee et al., 2013; Williams et al., 2001). A study by Fujiwara et al. (2018), using a human fetus-derived neural progenitor cell line (ReNcell) has shown that oestrogen at  $10^{-10}$ M and bisphenol A (an ER agonist/antagonist) at  $10^{-16}$  and  $10^{-10}$  M suppresses neural differentiation. Other studies have also documented BPA exposure suppresses synapse formation, disrupts neural migration, increases the number of glial cells, and upregulates neural cytoskeletal proteins (Fujiwara et al., 2018; Iwakura et al., 2010; Komada et al., 2012; Leranthe et al., 2008; Nakamura et al., 2007; Yamaguchi et al., 2006; Yokosuka et al., 2008).

### **1.2.2 The Androgenic pathway**

Androgens such as testosterone are sex steroid hormones that play a crucial role in neurodevelopment (ND). Androgen affects special brain regions and provides sexual differentiation of the central nervous system. During postnatal development, testosterone is present in cortical regions of the brain, and it is important for cognition (Kelemenova & Ostatnikova, 2008). It has been shown in humans that exposure to high levels of prenatal testosterone results in masculinized social behaviour in the areas of spatial performance and cognitive abilities (Knickmeyer et al., 2005).

Androgen controls the expression of target genes involved in processes such as neurotransmitter production and release, synapse conformation changes, control of neuronal apoptosis and alteration of neurochemical profiles (Alonso-Solís et al., 1996). Binding of androgens to their receptor (AR) results in receptor complex formation that



serves in the nucleus as a transcription factor (Kelemenova & Ostatnikova, 2008; Moilanen et al., 1998).

AR is the 4th member of the NR3C subgroup of a nuclear receptor superfamily mediating the action of steroid hormones (Auwerx et al., 1999). ARs are present in the hippocampus, amygdala, and prefrontal cortex, which are crucial for memory and learning (Beyenburg et al., 2000; Finley & Kritzer, 1999; Kelemenova & Ostatnikova, 2008). Some EDCs have the potential to act as agonists or antagonists of this receptor (e.g., Polychlorinated biphenyl (PCB), phthalates) thus disrupting the activity of endogenous androgens.

### **1.2.3 The thyroid hormone pathway**

Thyroid hormones (THs) play critical roles in foetal and post-natal nervous system development and the maintenance of adult brain function (Schroeder & Privalsky, 2014; Wallis et al., 2010). The TH requirement for development is most apparent in the central nervous system (CNS), and it mediates CNS effects primarily through the thyroid hormone receptors (TRs) (Schroeder & Privalsky, 2014).

The thyroid receptor is also a nuclear receptor; however, TR can bind to DNA in the absence of thyroid hormone to repress gene transcription. Upon hormone binding, a conformational change in the receptor causes it to activate transcription of target genes (Brent, 2012; Gouveia et al., 2020). As such, the TH receptor acts also as ligand-dependent transcription factor.

In comparison to thyroxine (T4), TRs have a 10-fold higher affinity for 3,5,3'-triiodothyronine (T3), making T3 the active thyroid hormone (Gouveia et al., 2020). There are four known isoforms of TR, which are encoded by THRA and THRB genes (Freedman, 1992). THRB encodes TR $\beta$ 1 and TR $\beta$ 2, while THRA encodes TR $\alpha$ 1 and TR $\alpha$ 2. These isoforms have similar DNA-binding domains but different ligand-binding and transactivation domains. For example, TR $\alpha$ 2 has unique carboxy terminus and so it does not bind to T3 (VIVO Pathophysiology 2020; Gouveia et al., 2020). TR- $\alpha$ 1 is the most abundantly expressed TR isoform in the human brain, accounting for 70–80% of total TR expression, and it is responsive to both T3 and T4 (Schroeder & Privalsky, 2014).

Several types of EDCs are structurally similar to TH, some of which are BPA, Dichlorodiphenyltrichloroethane (DDT), PCB, polybrominated biphenyls, kelthane, methoxychlor, nitrofen, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans (McKinney and Waller 1998). As such, there might be an interaction between EDCs and TH in the development of CNS (Koibuchi et al. 2003). TRs are susceptible to EDCs and their altered function can induce developmental neurotoxic effects in exposed organisms. Experimental studies in rats have shown that thyroid hormone regulates events during early brain development (e.g., neuronal migration in the cortex) (Naveau et al., 2014). Exposure to PCBs at concentrations commonly observed in humans can alter thyroid hormone receptor signalling (mainly through TR $\beta$  complex) and disturb oligodendrocyte differentiation and white matter maturation during early development (Fritsche et al., 2005). Miyazaki et al. (2004) stated that partial dissociation of the TR/retinoid X receptor heterodimer complex from the TH response element could be involved in the suppression of transcription induced by PCB (Masuo & Ishido, 2011).

Iwamuro et al. (2006) demonstrated that BPA acts as an antagonist of T3 through suppression of TR $\alpha$  and TR $\beta$  gene expression. The differentiation of mouse oligodendrocyte precursor cells is induced by T3 via TR $\beta$ 1, and this activity is inhibited by BPA (Seiwa et al. 2004). Zoeller et al. (2005) noted that exposure to BPA during pregnancy and lactation produces an increase in serum T4 and the expression of the TH-responsive gene RC3/neurogranin in the dentate gyrus of rat pups. Their findings showed that BPA antagonizes TR $\beta$ , which mediates the negative feedback effect of TH on the pituitary gland, but is less effective at blocking TR $\alpha$ , thus resulting in TR $\alpha$ -mediated events in response to elevated levels of T4.

### **1.3 Effects of EDCs on Neurodevelopment**

Epidemiological data supports associations between exposure to EDCs and neurodevelopmental disorders such as ADHD (attention deficit hyperactivity disorder), autistic disorder, learning and memory deficits (Bergman et al., 2012). Likewise, there is evidence indicating that the exposure of pregnant animals to EDCs leads to abnormalities in behaviour and brain functions of offspring. This is not far-fetched since hormones play critical roles in neurodevelopment and as such, neurodevelopmental disorders in humans and wildlife could be related to exposure to EDCs (Bergman et al., 2012). However, traditionally, research regarding adverse effects of EDCs has focused on the reproductive system, teratology, and metabolism (Masuo & Ishido, 2011).

Neuroimaging studies have shown brain morphological abnormalities (e.g., cortical thinning) consistent with neurodevelopmental impairments (e.g., impaired cognition, psychomotor and language development, behavioural problems) to be associated with exposure to EDCs (Ghassabian & Trasande, 2018). EDCs have also been shown to impair hippocampal functions, a brain region that plays important roles in learning and memory and it is sensitive to stressors. Kim et al. (2009) reported that treatment of mice with BPA (20 mg/kg bw) accelerated the formation of the dentate gyrus at postnatal day 1 without affecting neurogenesis in the same area. In vitro, BPA at high concentrations attenuated the proliferation of murine-derived multipotent neural progenitor cells and showed cytotoxicity. However, low concentrations of BPA stimulated the cells to differentiate into a neuronal phenotype. As such, BPA might stimulate neuronal differentiation and disrupt neonatal brain development (Masuo & Ishido, 2011).

The developmental stage at which the chemical exposure occurs will determine the nature and severity of the health outcomes (Masuo & Ishido, 2011). For example, Zsarnovszky et al. (2005) reported that a low dose of BPA affected the development of rat embryos and disrupted the activity of 17 $\beta$ -oestradiol in the development of the cerebellum. Thus, suggesting that BPA at low doses might influence cerebral development (Masuo & Ishido, 2011). EDCs can cross the placenta and the immature blood-brain barrier of embryos and neonates can be exposed to these chemicals directly from breast milk (Ikezuki et al., 2002; Mose et al., 2007; Oh et al., 2008). The alteration of molecular processes taking part during sensitive windows of brain development can lead to changes in cellular organization, differentiation, and response to external stimuli (Attoff et al., 2017; Bal-Price et al., 2015; Smirnova et al., 2014, Van Thriel et al., 2012). Hence, endpoints associated with neurodevelopmental toxicity include the disruption of biological events such as neurogenesis, neural differentiation, migration, apoptosis, synaptogenesis, neuritogenesis,

and the balance between gliogenesis and neurogenesis (Kalia, 2008; Somogyi et al., 2016). Also, subtle alterations in synapse number, connectivity, ratio of neural cells subpopulations, or cell positioning can cause developmental neurotoxicity (DNT) (Attoff et al., 2017; Bremer et al., 2010; Kadereit et al., 2011).

## **1.4 Developmental Neurotoxicity Testing**

### **1.4.1 Current Regulatory Status**

The current increase in the number of children diagnosed with learning and neurodevelopmental disorders, highlights the need for the development of methods to evaluate DNT induced by exposure to chemicals (Attoff et al., 2017; Herbert, 2010). The current available guidelines for DNT testing use in vivo models with endpoints such as behaviour, sexual maturation, brain weight and neuropathology (OECD 2007; US EPA 1998). However, this guideline is time consuming, expensive, and comes with ethical costs (Tsuji & Crofton, 2012). Due to the large amount of chemicals produced, there is a need for the development of rapid, high-throughput in vitro methods that can be applicable for the screening of DNT effects of chemicals (Attoff et al., 2017; Council, 2007; Fritsche et al., 2017).

### **1.4.2 The C17.2 cell line**

Established cell lines and primary cultures of mammalian cells are alternatives to the currently used animal models for neurotoxicity testing (Lundqvist et al., 2013). For neurotoxicity testing, a primary tissue culture of mixed cell types should be the best in vitro model, because glia cells tend to modulate neuron sensitivity to chemicals (Zurich et al., 2004). The cloned cell line method has not been able to mimic the brain complexity and its cell-to-cell communication (Forsby et al., 2009). Therefore, the C17.2 cell line, which is a multipotent neural progenitor cell line, was presented as an alternative to primary brain tissue culture (Attoff et al., 2017; Lundqvist et al., 2013). The C17.2 cell line was generated from neural stem cells of the external germinal layer of mouse cerebellum and immortalised by avian myelocytomatosis viral- related oncogene (v-myc) transfection (Lundqvist et al., 2013; Snyder et al., 1992). This cell line can be differentiated into a mixed culture of astrocytes and neurons using serum-free Dulbecco's modified essential medium F12 (DMEM:F12) with N2 supplements following a 10 days differentiation protocol (Attoff et al., 2017; Lundqvist et al., 2013). Biomarkers like nestin,  $\beta$ III-tubulin and glial fibrillary acidic protein (GFAP) can be used to validate the different cell types in culture for neural progenitor, neurons, and astrocytes, respectively (Lundqvist et al., 2013). Notably, the C17.2 cell line expresses a number of NRs, among others the ERs, AR, and THR. Therefore, this cell line has the potential to be used for screening of endocrine disruption (ED)-induced DNT.

## **1.5 Project aim**

This project focused on the optimization of the C17.2 cell line for the detection of EDC-induced DNT with emphasis on the disruption of the oestrogen, androgen, and thyroid hormone systems. This is aimed at validating the involvement of oestrogen, androgen, and thyroid hormone on molecular and cellular endpoints relevant for the differentiation of the neural progenitor C17.2 cells.

## 2.0 MATERIALS AND METHODS

### 2.1 Chemicals and reagents

List of chemicals and reagents, cas/reference number and manufacturer are presented as follow, Oestradiol (E2257), fulvestrant (14409), dihydrotestosterone (A8380), hydroxyflutamide (H4166) and triiodothyronine (T2877), were purchased from Sigma Aldrich. NH-3 (provided by Dr. Pam Lein (UC Davis, CA, USA)). PBS (10010023), DMEM (31053028), DMEM/F12 (11039021), HS (26050-088), FBS (10270), GIBCO® L-glutamine (25030-024), GIBCO® TrypLE™ Express enzyme (12604021) (12604013), GIBCO® Sodium pyruvate (11360039), GIBCO® N2 supplements (17502001), AlamarBlue® cell viability reagent (DAL1100) and Hoechst stain 33342 (H3570) were purchased from Thermo-Fisher Scientific (Sweden). Poly-L-Lysine solution (P4707) and Triton X-100 (93443) from Sigma Aldrich (Sweden). BSA (9048468) from USB corporation (Cleveland, USA). DMSO (A3672,0050) PanReac AppliChem ITW Reagent (Sweden). Formaldehyde solution 4% (1004968350) from Merck KGaA (Germany). CytoTox 96® (G1780) from Promega. RNA extraction kit RNeasy® Mini Kit (74104 and 74106) was purchased from Qiagen. iScript gDNA clear cDNA synthesis kit (172-5035) and SsoAdvanced™ Universal SYBR Green Supermix (1725270) for RT-qPCR were purchased from Bio-Rad (Sweden).

### 2.2 Cell-line, cell culturing and differentiation

The neural progenitor cell line C17.2 was a generous gift from Professor Sandra Ceccatelli (Karolinska Institutet, Stockholm, Sweden), with the permission of Professor Evan Snyder (Harvard Medical School, Boston, USA). The C17.2 cell line was cloned from neural stem cells of the external germinal layer of postnatal day 4 mouse cerebellum, immortalized by avian myelocytomatosis viral- related oncogene (v-myc) transfection (Lundqvist et al., 2013; Snyder et al., 1992).

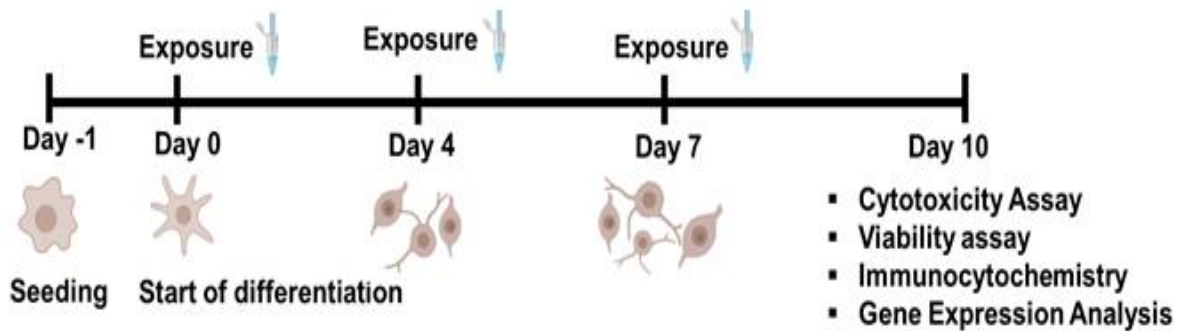
#### *Routine culture*

C17.2 cells were grown at a density of 1180 cells/cm<sup>2</sup> in 100 mm cell culture dishes (430591, Corning Inc, Corning NY) and kept at 37°C in an 5% CO<sub>2</sub> incubator. A low cell density was used because the cells are known to spontaneously differentiate if the culture becomes too dense. Also, the passage of all cells used for this study was lower than 29, because it was shown that the cells do not maintain stable karyotype at passages over 30 (Lundqvist et al., 2013). Undifferentiated cells were kept and propagated in DMEM medium supplemented with 10% FBS, 5% HS, 1% sodium pyruvate and 200 mM L-glutamine, referred to as routine culture medium from now on. For routine passaging, the cells were detached every 3.5 days using TrypLE™ Express enzyme and subcultured in a new cell culture dish at the same density as above. At each passage, cell concentration and viability were determined by counting with an automatic cell counter (EVE™, NanoEnTek), using trypan blue stain 0.4% (T10282; Invitrogen, CA, USA).

#### *Differentiation*

C17.2 cells were seeded in 0.01% Poly-L-lysine coated 96 or 6 well culture plates at a density of 1250 cells/cm<sup>2</sup>. Twenty-four hours after seeding, the routine culture medium was removed and replaced with differentiation medium (DMEM/F-12 medium

supplemented with 200 mM L-glutamine and modified N2 supplements). Due to the autocrine function of the cells, they secrete nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF), so they can differentiate into a co-culture of neurons and neuroglia in the absence of serum. Half of the culture medium was renewed every 3rd day for the 10-day differentiation period (Fig. 1) and replaced with fresh differentiation medium containing either DMSO 0.1% (negative control), or the receptor agonist and antagonist dilutions as the case maybe. The cells were kept in a humidified atmosphere of 5% CO<sub>2</sub> and 37°C in an incubator.



**Figure 1:** Schematic overview of the differentiation and exposure procedure

## 2.3 Determination of non-cytotoxic concentrations

### 2.3.1 Exposure to the receptor's agonists and antagonists

To establish the non-cytotoxic concentration of the hormonal agonist and antagonist to be used for assessment of morphological response of the cell and differential expression of the marker genes, the C17.2 cells were seeded with 100µl/well routine culture medium in a Poly-L-lysine coated, transparent 96 well cell culture plate (10062-900; VWR®, VWR International) at a density of 1250 cells/cm<sup>2</sup>. Twenty-four hours after seeding, the routine culture medium was changed to differentiation medium (100µl/well), before exposure. Prior to exposure, all receptor agonists and antagonists were dissolved in 100 % DMSO. They were then freshly diluted to the different concentration ranges in differentiation medium, following a serial dilution ratio of 1:5 (Table 1). For exposure, 100 µl/well of each hormone dilution were added to each well in three technical replicates. A solvent control, containing 0.1% DMSO in differentiation medium was added to each plate (nine technical replicates per plate). Half of the medium was changed every 3rd day and renewed with appropriate hormone dilution throughout the 10 days' exposure and differentiation period (Fig. 3).

**Table 1: Concentration ranges of receptor agonist and antagonist tested for all experiments.**

Experiment	Receptors	Agonist	Concentrations (nM)	Antagonist	Concentrations (nM)
LDH and viability assay	ER	Oestradiol	<sup>a</sup> 2500 to 3.2E-02	Fulvestrant	<sup>a</sup> 500 to 6.4E-03
	AR	Dihydrotestosterone	<sup>a</sup> 2500 to 3.2E-02	Hydroxyflutamide	<sup>a</sup> 2500 to 3.2E-02
	TR	Triiodothyronine	<sup>a</sup> 5000 to 6.4E-02	NH-3	<sup>a</sup> 5000 to 1.6E-07
Immunocytochemistry	ER	Oestradiol	<sup>a</sup> 2500 to 3.2E-02	Fulvestrant	<sup>a</sup> 500 to 6.4E-03
	AR	Dihydrotestosterone	<sup>a</sup> 2500 to 3.2E-02	Hydroxyflutamide	<sup>a</sup> 2500 to 3.2E-02
	TR	Triiodothyronine	<sup>a</sup> 5000 to 6.4E-02	NH-3	<sup>a</sup> 200 to 2.57E-03
Gene validation	ER, AR, TR	Oestradiol Dihydrotestosterone Triiodothyronine	<sup>b</sup> 100, 10, 1	Fulvestrant Hydroxyflutamide NH-3	<sup>b</sup> 100, 10, 1

Note: The concentration dilution factor is <sup>a</sup>1:5, <sup>b</sup> 1:10. ER-oestrogen receptor, AR-androgen receptor, TR-Thyroid receptor.

### 2.3.2 Cell viability and Cytotoxicity assay

After the 10-days exposure and differentiation period, the effect of the receptors' agonist/antagonist on cell viability and cytotoxicity was determined.

#### *Cytotoxicity assay*

This was carried out using CytoTox 96® non-radioactive cytotoxicity assay reagents as described by the manufacturer. In brief, each well contains 200 µl medium and cell after the 10days exposure and differentiation period. 50 µl/well aliquots of these mediums were transferred to a sterile clear flat bottom 96-well plate to measure lactate dehydrogenase (LDH) released in the medium. To estimate the cytotoxic effect of the tested hormones, the total LDH content of the cells was measured by lysing the cells. For this, another 50 µl were removed from all wells leaving 100 µl in each well. 10 µl/well lysing solution was added to all wells and incubated for 45 minutes at room temperature. After incubation, 50 µl/well aliquots of the medium were transferred to a sterile clear flat bottom 96-well plate to measure Maximum LDH. To the LDH in the medium (referred as LDH release) and Maximum LDH plates, 50 µl/well CytoTox 96® reagent was added, and the assay was stopped with a stop solution after 30 minutes' incubation at room temperature. The absorbance was then read at 490nm with a microplate reader (Perkin Elmer VICTOR<sup>3</sup> 1420 Multilabel counter). Percentage of cytotoxicity and percentage cytotoxicity relative to control were estimated from the generated data.

$$\% \text{ Cytotoxicity} = 100 \times (\text{LDH release} / \text{Maximum LDH release})$$

#### *Cell viability assay*

The AlamarBlue cell viability reagent, which measures cellular metabolic activity, was used to determine the number of viable cells. 110 µL/well aliquots of test and control medium were removed from the assay plate. To the remaining 90 µL, 10 µL/well of the AlamarBlue reagent were added and the plates were incubated (at 5% CO<sub>2</sub> and 37°C) for 2 hours. After incubation, fluorescence was read at 570 nm excitation and 620 nm emission using Tecan Spark® multimode microplate reader. Percentage metabolic activity of the exposed cells relative to the negative control was then determined. The inhibitory

concentration 10% (IC10) was determined as described by Attoff et al. (2017) using nonlinear regression with log(inhibitor) vs response (variable slope), equation.

$$Y = \text{Bottom} + (\text{Top}-\text{Bottom})/(1+10^{((\text{LogIC10}-X) * \text{HillSlope}))} \text{ (GraphPad Prism 5)}.$$

Both assays were carried out in three to four biological replicates, each of which included three technical replicates.

## **2.4 Immunocytochemistry, cell imaging and image analysis**

To assess the effect of the hormonal pathways on the differentiation potential and morphology of the C17.2 cell-line, the cells were exposed to the receptor's agonist and antagonist (Table 1), 0.1% DMSO (negative control) and 125 nM rotenone (positive control), for the 10 days differentiation period, using a sterile black, transparent, and flat bottom 96-well plate (3603; Corning Inc. Corning NY). At the end of the exposure, 100  $\mu$ l of the culture medium were removed from each well and the cell nuclei were stained with 20  $\mu$ l/well Hoechst staining solution (20mM) for 20 minutes, at 5% CO<sub>2</sub> in air and 37°C. The cells were then fixed by adding 120  $\mu$ l/well 4% paraformaldehyde (PFA) to all wells giving a final concentration of 2%PFA. The cells were then incubated for 20 minutes at room temperature. After washing twice in 200  $\mu$ l PBS, cells were permeabilized and blocked with 100  $\mu$ l/well of 2% BSA/PBS in Triton X-100 (0.5 %) overnight at 4°C. Subsequently, the cells were incubated with 100  $\mu$ l/well Rabbit polyclonal  $\beta$ III-tubulin primary antibody (1:1000 dilution) (GR3300802-1; Abcam, Netherlands), for 1h at room temperature. After incubation, the cells were washed twice in 200  $\mu$ l 2% BSA/PBS and incubated for 30 minutes in the dark, at room temperature with 100  $\mu$ l/well Goat Anti-Rabbit IgG H&L secondary antibody (1:400 dilution) (GR3313703-1; Abcam, Netherlands). They were then washed twice and preserved with PBS 200  $\mu$ l/well. The images were captured using ImageXpress micro (molecular devices) with a 10  $\times$  objective lens, and 9 sites per well were imaged. The fluorescence from the  $\beta$ III tubulin-stained cell bodies and neurites was detected in the FITC channel (excitation  $475 \pm 34$  nm, emission  $536 \pm 40$  nm). Nuclei were identified on the DAPI channel (excitation  $377 \pm 50$  nm, emission  $447 \pm 60$  nm).

Image analysis was carried out to estimate the total number of cells, percentage ratio of neurons in culture, and neurite outgrowth per well for the different exposure concentration and treatments, using CellProfiler software as described by Jones et al. (2008) and MetaXpress® software (Molecular Devices). For image analysis with CellProfiler software (Appendix 1A-B), individual images were manually selected, discarding those out of focus by using ImageJ software to view the images. The CellProfiler pipelines were designed using modules to identify primary objects (nuclear staining with Hoechst 33342) at 21-54 pixel using global and minimum cross-entropy threshold strategy. The secondary objects ( $\beta$ III tubulin-stained cell bodies and neurites) were identified using propagation, adaptive, Otsu threshold strategy. Neurite outgrowth was estimated as total cytoskeleton using the measure object skeleton module.

Briefly, for the MetaXpress® software analysis (Appendix 1A-B), all images were analysed without removing images that are out of focus, because the software is sensitive enough to analyse them. The mean neurite length was calculated using the Neurite

Outgrowth plug-in in the software. Outgrowths were defined as cytoplasmic prolongations with a maximum width of 2  $\mu\text{m}$  (3 pixels). The minimum outgrowth size needed to be logged as significant was defined as 20  $\mu\text{m}$  (31 pixels). Nuclear staining with Hoechst 33342 was determined using the Cell Scoring plug-in in the software, for nuclei with a size between 10 – 50  $\mu\text{m}$  (15-77 pixels). Visual examination of the images was also carried out with the aid of ImageJ software (version 1.8), to assess for proportion of neuron to cell and observable neurite impairment of the cells in response to the agonist/antagonist treatment. Random manual counting of cells and neurons with the negative control images from each treatment was also carried out using the cell counter plug-in in ImageJ software as described by Pemberton et al. (2018) (Appendix 1C).

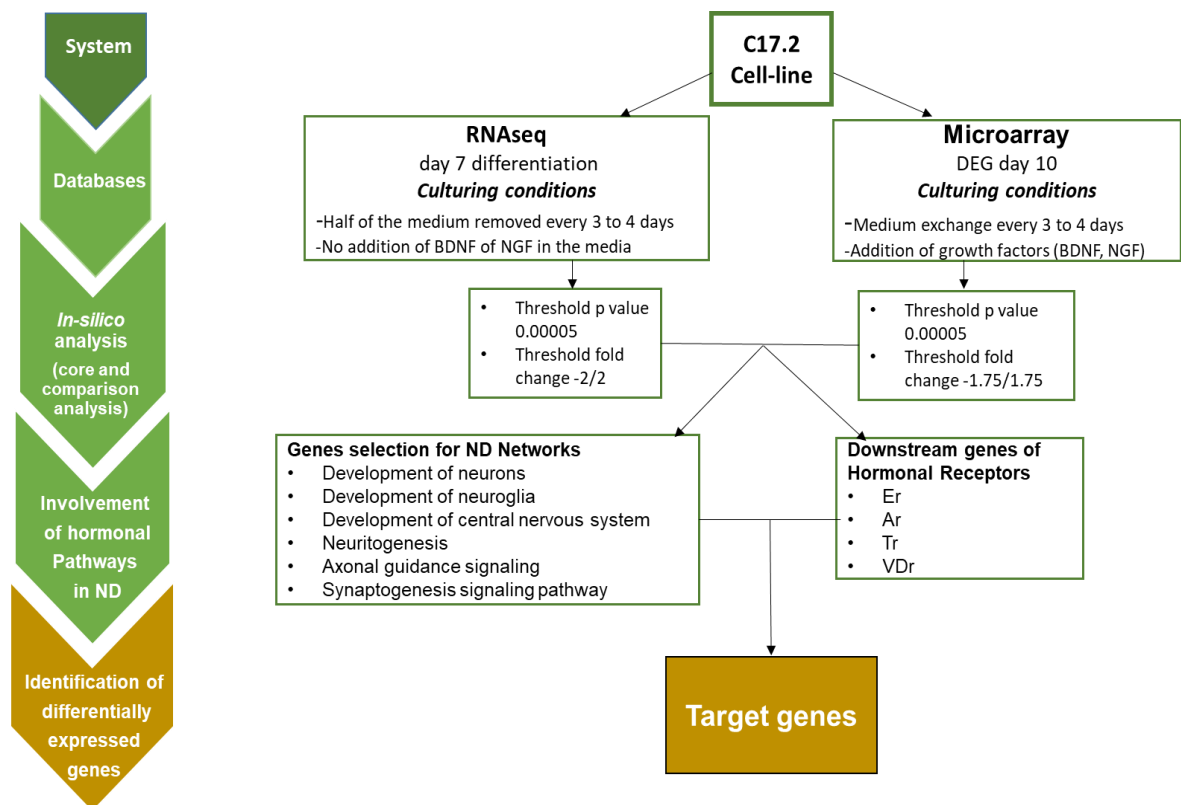
## 2.5 *In-silico* analysis

In order to identify genes regulated by each of the hormonal receptors and at the same time necessary for neurodevelopment in the C17.2 cells, *in-silico* analysis of transcriptomic data was carried out using the Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, Redwood City, CA). Downstream analysis was used to identify differentially expressed genes (DEGs) involved in neurodevelopment (ND) via the hormonal pathways (Fig. 2). RNA sequencing (RNA-seq) data of 7 days differentiated C17.2 cells from Gliga et al. (2017) and microarray data of 10 days differentiated C17.2 cells from Attoff et al. (2017) were used for this analysis. To identify genes involved in ND, core and comparison analysis were carried out at a p-value < 0.000005 with a fold change threshold equal or higher than  $\pm 2$  for the RNA-seq data, and equal or higher than  $\pm 1.75$  for the microarray data. This was carried out to map out genes involved in ND networks such as development of neurons, development of neuroglia, development of the central nervous system, neuritogenesis, axonal guidance signalling, and synaptogenesis signalling pathway. Upon removal of duplicate genes, there were a total of 160 genes involved in ND in the C17.2 cell line. Subsequently, upstream regulator analysis of the hormonal receptor (ER, AR and TR) pathways was carried out. This was carried out to map out downstream ND genes, regulated by these receptors and present in the RNA-seq and microarray data. The gene outputs were screened using a fold change higher than 2 or lower than  $-2$  for RNAseq data and a fold change higher than 1.75 or lower than  $-1.75$  for microarray data. Upon removal of duplicates there were a total of 53 genes for AR, 82 for ER and 13 for TR.

Venn diagrams of the DEGs associated with ER, AR and TR pathways and neurodevelopment networks, were plotted using the web tool from the Bioinformatics & Evolutionary Genomics Laboratory at VIB/UGent, Belgium (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) (Attoff et al. 2017).

The identified DEGs regulated by each of the hormonal receptors, necessary for neurodevelopment, were then used to generate a heatmap. The DEGs were further validated by RT-qPCR, using mouse specific primers designed using PrimerBank (<https://pga.mgh.harvard.edu/primerbank/>) and the National Centre for Biotechnology Information (NCBI; U.S. National library of medicine) and procured from Sigma Aldrich (Sweden).





**Figure 2:** Flowchart of in-silico analysis for the identification of ND genes regulated by endocrine pathways.

## 2.6. Validation of selected DEGs

### 2.6.1 mRNA extraction

To validate the occurrence of the identified DEGs in the C17.2 cells, the cells were differentiated and exposed to DMSO 0.1%. After the 10-day differentiation period, culture medium was removed, cells were washed once with PBS, and then harvested using a 600 µl/well RLT buffer from the Qiagen RNeasy Mini Kit. Cells were then kept at -80°C to lyse for at least 24hrs before mRNA isolation. The extraction was carried out following manufacturer's instructions in the Qiagen RNeasy Mini Kit. Briefly, 1 volume (600 µl) of 70% ethanol was added to the lysate and 700 µl of this was transferred to a RNeasy Mini spin column with 2 ml collection tube, and centrifuged for 15 seconds at 8000 g. Afterwards, the flow-through was discarded, 700 µl Buffer RW1 and 500 µl RPE were added in sequential order to the column and centrifuged for 15 seconds at 8000 g. 500 µl RPE were then added to the column and centrifuged for 2 minutes at 8000 g. This was then centrifuged at full speed for 1 minute to dry the column membrane. RNA elution was carried out using 50 µL RNase-free water for primer efficiency experiments or 30 µL RNase-free water for qPCR validation of selected DEGs, to the column membrane and centrifuge for 1 min at 8000 g. RNA concentration was determined using NanoDrop™ 2000C spectrophotometer (Thermo scientific) or Tecan Spark® multimode microplate reader.

### 2.6.2 Reverse Transcription Real-Time Quantitative Polymerase Chain Reaction

Following RNA isolation, 1-0.25 µg of the total RNA was reverse transcribed into cDNA using iScript gDNA Clear cDNA Synthesis Kit from Bio-Rad, following manufacturer instruction, with the aid of T100™ Thermal Cycler (Bio-Rad). In brief, to remove genomic DNA, appropriate volume of DNase master mix was prepared using iScript DNase and iScript DNase Buffer. 2 µl of the DNase master mix was added to 1 µg (for primer efficiency testing) or 250 ng (for qPCR validation of DEGs) of RNA diluted in 14 µl of RNase free water and set in the thermal cycler using a reaction protocol of 25°C, 5 minutes for DNA digestion and 75°C, 5 minutes for DNase inactivation. To synthesize cDNA, 4 µl of iScript Reverse Transcription Supermix was immediately added to the DNase-treated RNA template. The reaction was completed by incubating with the thermal cycler at 25°C, 5 minutes for priming, 46°C, 20 minutes for reverse transcription (RT), 95°C, 1 minutes for RT inactivation and held for 4°C until further use. To check for gDNA contamination in the RNA sample, a no-RT control was included by replacing iScript Reverse Transcription Supermix with iScript No-RT Control Supermix in the reaction.

For primer efficiency testing, serial dilutions of the cDNA were prepared using a 1:5 dilution ratio (50, 10, 2, 0.4, 0.08, 0.016 ng) of the cDNA. These cDNA dilutions were used alongside the no-RT control, negative control (water), and each designed primer for the RT-qPCR process to test primer efficiency. The RT-qPCR was performed with SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad) as described by the manufacturer, using a C1000™ Thermal Cycler (CFX384™ Real-Time System; Bio-Rad). This was carried out for 40 cycles according to the following: Polymerase activation step at 95°C for 2 minutes, followed by DNA denaturation step at 95°C for 5 seconds, an annealing step of 60°C for 30 seconds and melt curve 65-95°C for 5 seconds. The primer samples were normalized against 2 selected reference genes that were not differentially expressed during differentiation: TATA box binding protein (Tbp), and ribosomal protein lateral stalk subunit P1 (Rplp1). Generated data were analysed with the CFX Maestro software 1.1 (Bio-Rad). The mean CT for each primer and cDNA dilution series (in logarithms) were used to plot standard curves. The generated slope and Pfaffl (2001) efficiency equation were used to determine efficiency value (E).

$$E = 10^{(-1/\text{slope}) - 1} \quad \text{Pfaffl (2001)}$$

A primer is considered efficient if the E value is in the range of 0.6 – 1.1 and standard curve  $R^2$  is greater than 0.989. The primer specificity was also determined by the alignment of the melt curve.

### 2.6.3 Exposure to receptor's agonist and antagonist for validation of selected DEGs

To validate the DEGs as biomarkers of DNT in the C17.2 cell line, exposure and differentiation were carried out for the receptor's agonist and antagonist. After careful consideration of the non-cytotoxic but effective concentrations generated from the viability, cytotoxicity assays and immunocytochemistry image analysis, in conjunction with the documented effective concentration of each chemical, the concentrations 100 nM, 10 nM, and 1 nM of each receptor's agonist or antagonist and two negative controls (0.1%) were used, in three biological replicates (Table 1). The cells were seeded with 2ml/well routine culture medium, in a 6 well sterile cell culture plate (3736; Costar, Corning Inc) at a density of 1250 cells/cm<sup>2</sup> and were differentiated and exposed as described above. At the

end of the 10 days' differentiation and exposure period, the cells were washed in PBS 1ml/well, harvested and lysed with an RLT buffer (600 µl/well) from the Qiagen RNeasy Mini Kit, collected and placed at -80 °C for at least 24 hours before RNA extraction. After 24hrs, mRNA extraction, cDNA synthesis and RT-qPCR were carried out as discussed earlier with the selected efficient primers (Appendix 2).

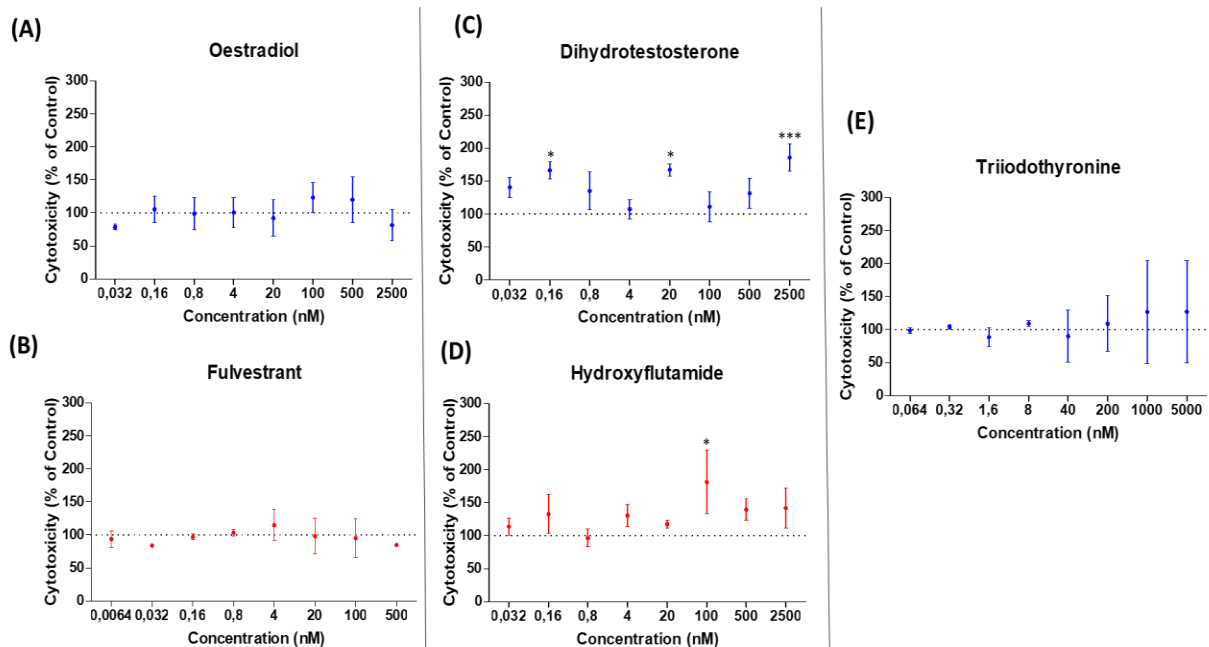
## 2.7 Statistical analyses

All the plots and statistical analysis were run using GraphPad Prism 5 (GraphPad software, CA, USA). The results are presented as mean  $\pm$  SD. Data were analysed using two-way ANOVA followed by Bonferroni post-test, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  to compare treatments to control.

## 3.0 RESULTS

### 3.1 Cytotoxicity and viability

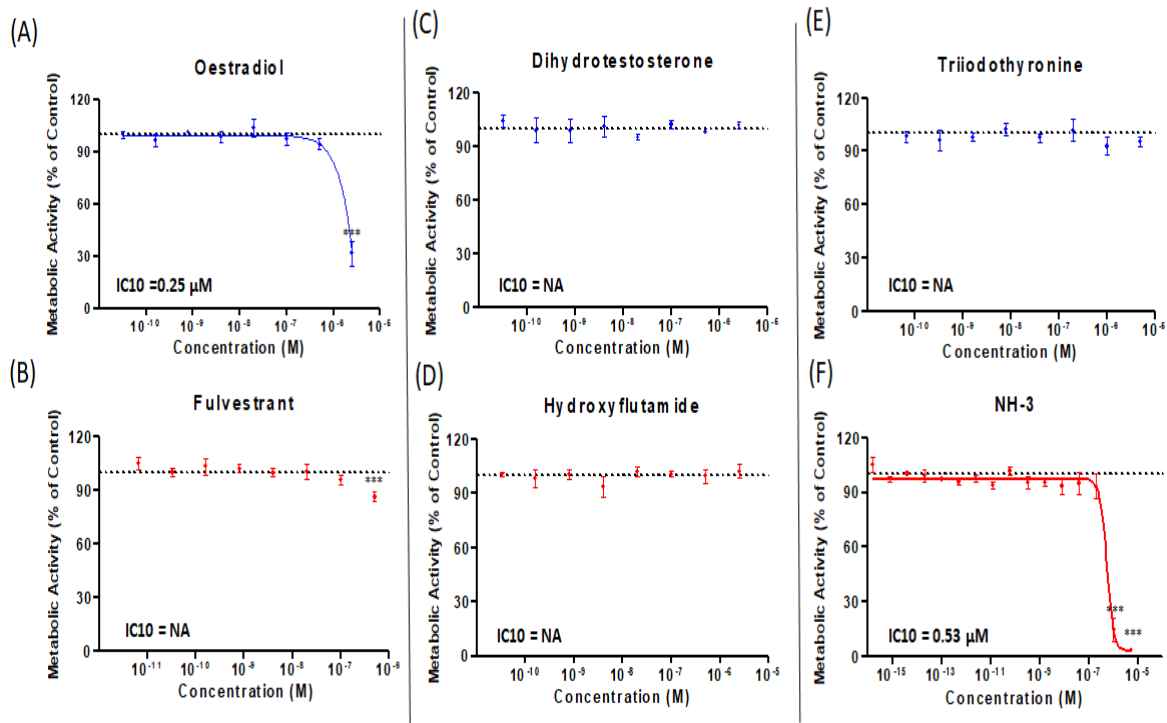
First, the non-cytotoxic concentration range of the hormone receptor agonists and antagonists to be used for morphological and gene expression analysis was assessed. Both cytotoxicity (measured LDH release from dead cell) and cell viability (measured metabolic activity of living cells) were assessed. After 10 days of differentiation and exposure period, cytotoxicity assay was carried out, and the result showed no significant difference between the tested concentrations and control for ER and TR agonist/antagonist treatment (Fig. 3A, B, E).



**Figure 3. Cytotoxicity of ER, AR and TR agonist and antagonist on the C17.2 cell-line.** Data are presented as a percentage of control (cells exposed to 0.1% DMSO). The cells were exposed to agonist (blue dot) and antagonist (red dot) of each receptor. (A) oestrogen (B) fulvestrant (C) dihydrotestosterone (D) hydroxyflutamide (E) triiodothyronine. Data are presented as the mean of three independent experiments performed in triplicates  $\pm$  SD. Data was analysed using two-way ANOVA with Bonferroni post-test, \*  $P \leq 0.005$ , \*\*\* $p \leq 0.001$ .

However, for AR agonist, cytotoxicity was observed at 0.16 nM, 20 nM and 2500 nM, while for the antagonist at 100 nM (Fig. 3C and D). Also, a wide standard deviation at the four highest concentrations was observed, compared with the other concentration tested (Fig. 3). The TR antagonist NH-3 was not tested because of delayed delivery and the cell response to the LDH assay. In summary, the result showed no cytotoxicity induced by the tested hormonal concentrations.

Cell viability assay was performed after 10 days differentiation and exposure period and IC<sub>10</sub> was estimated using the equation described above. For the oestrogen receptor pathway, the agonist oestradiol, at the highest concentration (2.5  $\mu$ M), significantly reduced the cell metabolic activity to 30% compared with control (Fig. 4A). The calculated IC<sub>10</sub> for oestradiol exposure in the cell was 2.5  $\mu$ M. Exposure to the ER antagonist fulvestrant reduced metabolic activity of the cells to 86% at 0.5  $\mu$ M (Fig. 4B), and this difference was statistically significant in comparison to the negative control ( $p=0.001$ ). On the other hand, for the androgen receptor, metabolic activity of the cells upon exposure to dihydrotestosterone (agonist) and hydroxyflutamide (antagonist) was not significantly different from the control at the tested concentrations (Fig. 4C-D). Thus, the IC<sub>10</sub> could not be computed for the AR agonist and antagonist. Similarly, metabolic activity of the cells upon exposure to thyroid receptor agonist (triiodothyronine) were not significantly different from the control at the tested concentrations (Fig. 4E). However, response to the thyroid hormone antagonist (NH-3), showed a statistically significant ( $p=0.001$ ) reduction of the metabolic activity in comparison to the control at 1  $\mu$ M and 5  $\mu$ M, where metabolic activity was reduced to 13.6 % and 1.8% respectively (Fig. 4F). The estimated IC<sub>10</sub> of the cells in response to NH-3 was 0.53  $\mu$ M.



**Figure 4. Viability of C17.2 cell-line upon exposure to varying concentrations of ER, AR and TR agonist and antagonist.** Data are presented as a percentage of control (cells exposed to 0.1% DMSO). The cells were exposed to agonist (blue dot) and antagonist (red dot) of each receptor. (A) oestrogen (B)

fulvestrant (C) dihydrotestosterone (D) hydroxyflutamide (E) triiodothyronine and (F) NH-3 viability. Data are presented as the mean of three independent experiments performed in triplicates  $\pm$  SD. Data was analysed using two-way ANOVA with Bonferroni post-test, \*\*\* $p \leq 0.001$ . The inhibitory concentration 10% (IC10) was determined using nonlinear regression with log(inhibitor) vs response (variable slope), equation.

### **3.2 Effects of hormone receptor agonists and antagonists on morphological endpoints**

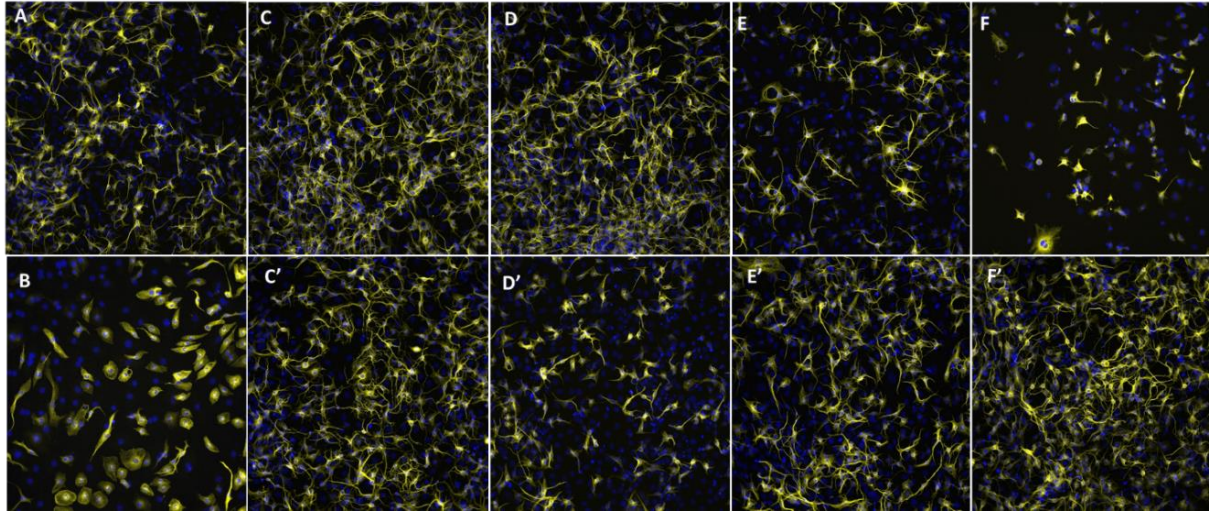
To determine whether the agonist and antagonist treatment is inducing morphological changes (percentage ratio of neurons in culture and neurite outgrowth) in the C17.2 cells, immunocyto staining was carried out. After 10 days differentiation and exposure of the C17.2 cells to the different concentrations range of agonists or antagonists, the cells were stained with  $\beta$ III-tubulin (neuron marker) and Hoechst (cell nucleus marker) for image analysis.

From the image analysis using CellProfiler, a 30 % neuron to cell in culture for all experimental negative controls was observed, while for MetaXpress an average of 16 % neuron to cell in culture was observed. However, image analysis of the negative controls by manual counting using imageJ software, showed 9 % neuronal cells in the culture.

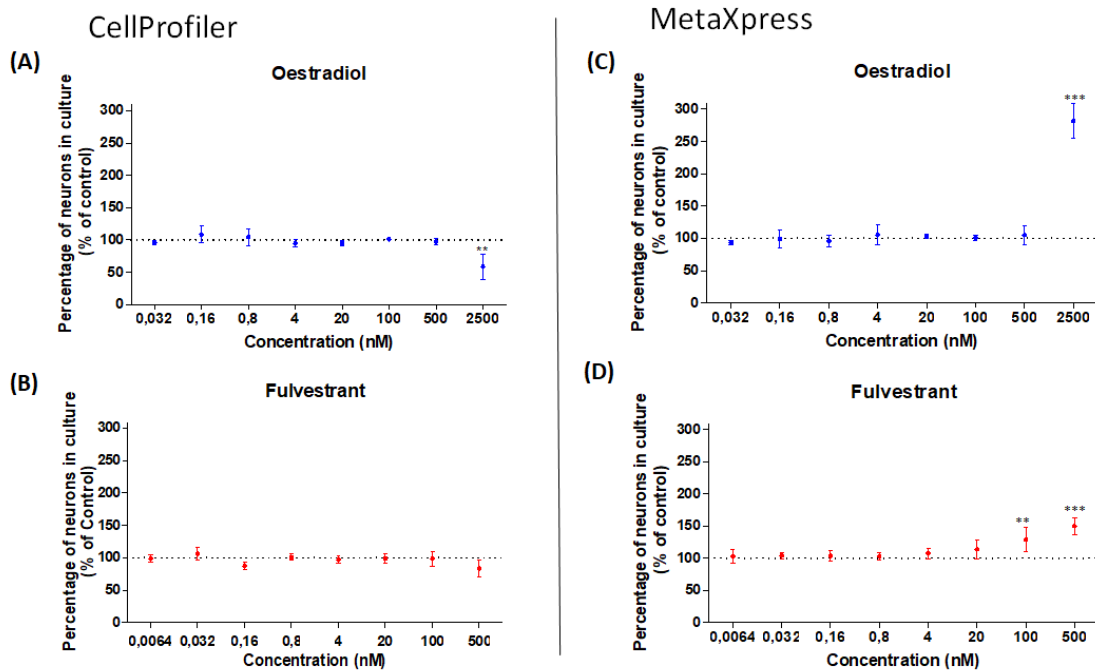
#### **3.2.1. Effects of ER agonists/antagonists on morphological neural differentiation endpoints**

Visual inspection of captured images for ER agonist (oestradiol) showed a reduction in neuronal cell number and neurite outgrowth with increase in concentration (Fig. 5C-F). The neuron morphology at the highest concentration (2500 nM; Fig. 5F) was similar to that observed in the positive control (Fig. 5B), having impaired neurite outgrowth. Further image analysis using CellProfiler (Fig. 6A) showed a decrease percentage of neurons in culture at 2500 nM, while the MetaXpress (Fig. 6C) showed an increase. Each software indicates a decrease in neurite outgrowth with increase concentration, which was significant ( $p=0.001$ ) at 2500 nM compared to control (Fig. 7A and C).

On the other hand, the response of the cells to the ER antagonist (fulvestrant) with visual examination depicts an increase in cell count with concentration (Fig. 5C'-F'; 6D-F). At 0.0064 nM, thinning and impairment of the neurite outgrowth was observed (Fig. 5C'). However, Image analysis of the cells with CellProfiler (Fig. 6B) showed a no significant changes in the percentage neurons in culture, while MetaXpress (Fig. 6D) showed significant ( $p<0.05$ ) increase in percentage neurons in culture with increase concentration. Each software also indicates no significant difference in neurite outgrowth with increase concentration of fulvestrant compared to control (Fig. 7B and D).

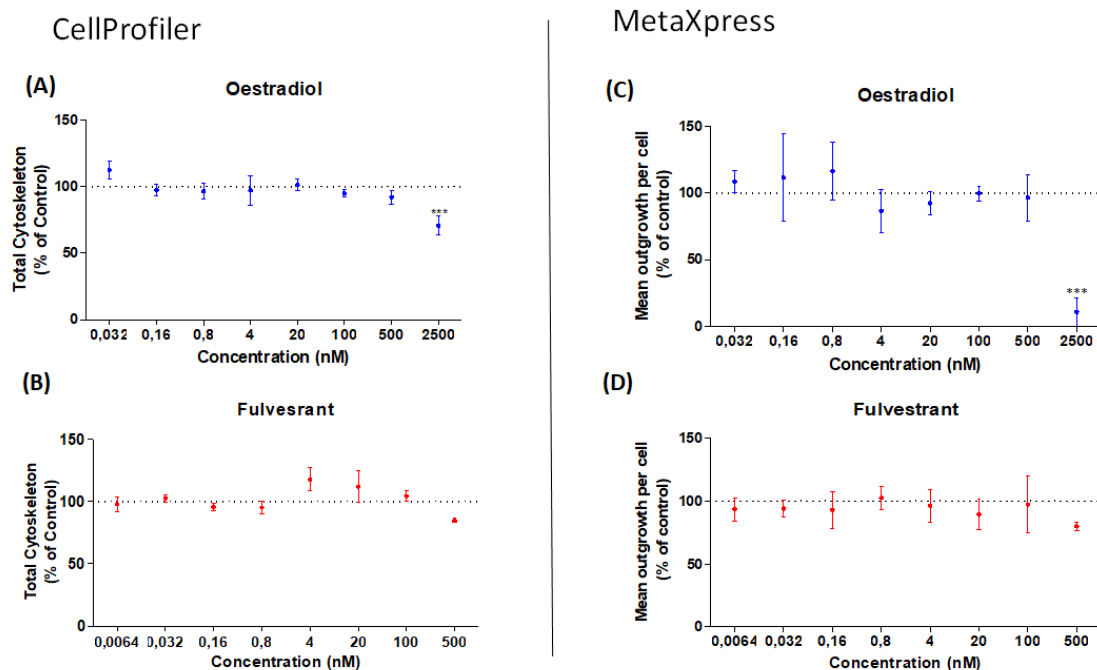


**Figure 5. Morphological changes in C17.2 cells after treatment with ER agonist and antagonist.** After 10 days differentiation and exposure, the cells were immuno-stained with  $\beta$  III-tubulin antibody (yellow) for neurons, and the nuclei were stained with Hoechst 33342 staining (blue) (A) 0.1 % DMSO-negative control (B) Rotenone –positive control (125 nM) (C-F) 0.8, 20, 100, 2500 nM oestradiol (C'- F') 0.0064, 0.8, 20, 500 nM fulvestrant.



**Figure 6. Percentage neurons in culture of the C17.2 cell-line on exposure to ER agonist and antagonist.** The Data generated from image analysis with CellProfiler and MetaXpress are presented as a percentage of negative control (cells exposed to 0.1% DMSO). The cells were exposed to agonist (blue dot) and antagonist (red dot) of the receptor. (A) Oestradiol effect on percentage neurons from CellProfiler (B) Fulvestrant effect on percentage neurons from CellProfiler (C) Oestradiol effect on percentage neurons from MetaXpress (D) Fulvestrant effect on percentage neurons from MetaXpress. Data are presented as the mean of three independent experiments performed in triplicates. Bars represent mean  $\pm$  SD. Data was analysed using two-way ANOVA with Bonferroni post-test, \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ .



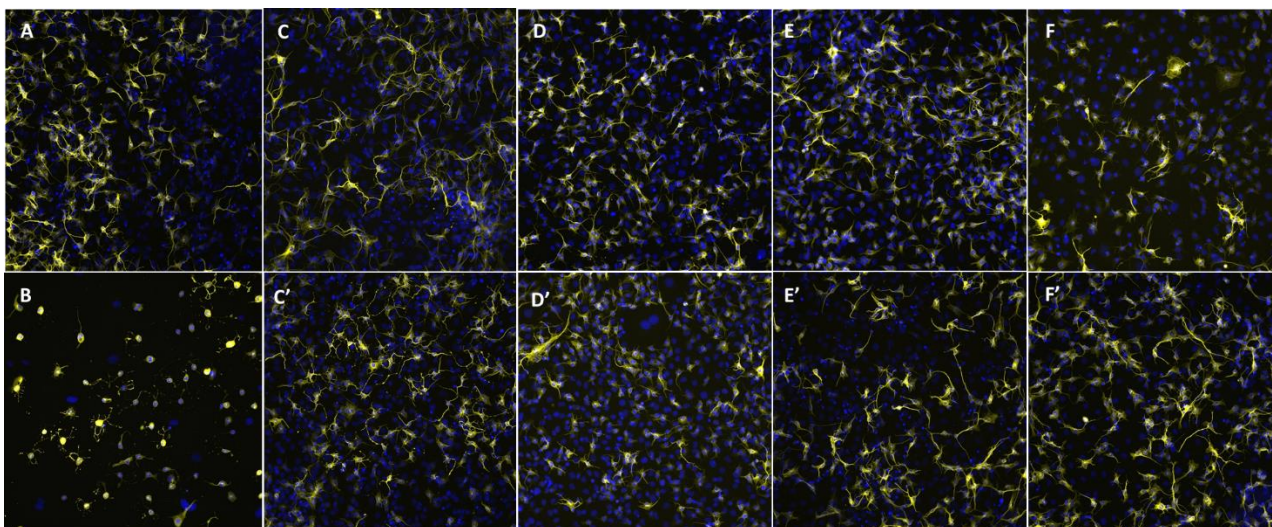


**Figure 7. Neurite outgrowth of the C17.2 cell-line on exposure to ER agonist and antagonist.** The Data generated from image analysis with CellProfiler and MetaXpress are presented as a percentage of negative control (cells exposed to 0.1% DMSO). The cells were exposed to agonist (blue dot) and antagonist (red dot) of the receptor. (A) Oestradiol effect on total cytoskeleton from CellProfiler (B) Fulvestrant effect on total cytoskeleton from CellProfiler (C) Oestradiol effect on mean outgrowth per cell from MetaXpress (D) Fulvestrant effect on mean outgrowth per cell from MetaXpress. Data are presented as the mean of three independent experiments performed in triplicates. Bars represent mean  $\pm$  SD. Data was analysed using two-way ANOVA with Bonferroni post-test, \*\*\* $p \leq 0.001$ .

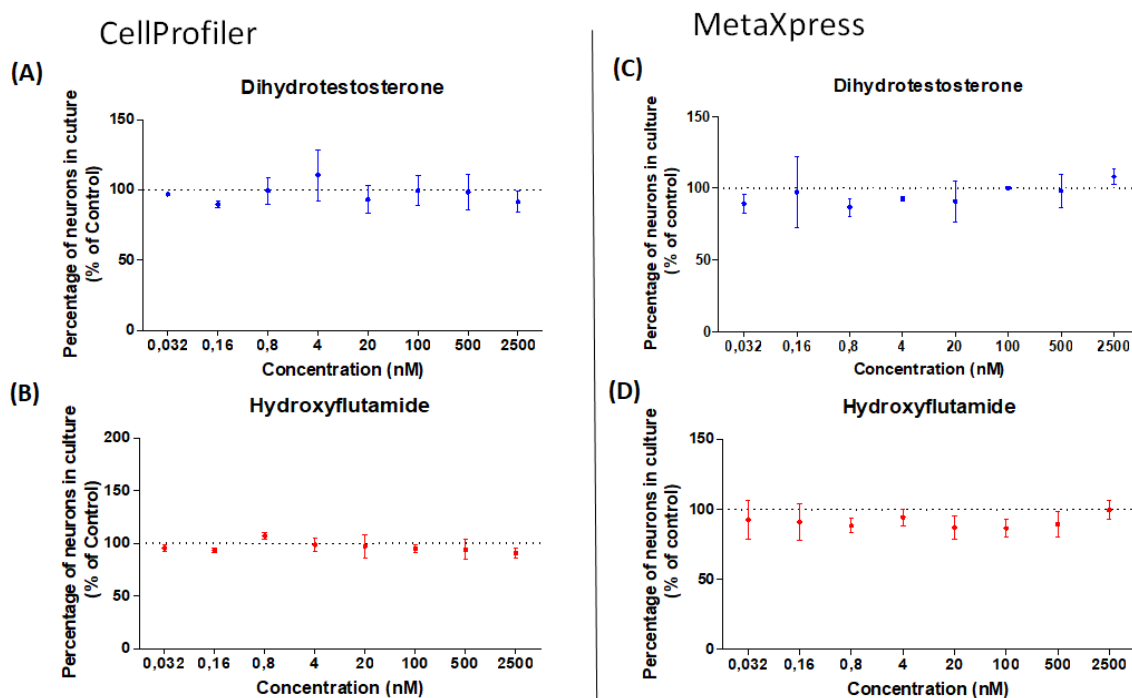
### 3.2.2. Effects of AR agonists/antagonists on morphological neural differentiation endpoints

Androgenic effects on C17.2 cells showed thinning and impairment of neurite outgrowth with increase in the concentration of dihydrotestosterone (Fig. 8C-F). The phenotypic response at the highest concentration tested (500 nM; Fig. 8F) was similar to that observed in the positive control (Fig. 8B), having impaired neurite outgrowth. However, image analysis with both software did not report significant changes in the percentage of neurons in culture and neurite outgrowth of the exposed cells in comparison to the control (Fig. 9A and C, 10 A and C).

Similarly, for the antagonist (hydroxyflutamide) the cells showed impairment of neurite outgrowth in response to the antagonist treatment (Fig. 8C'-F'), especially at 0.16 nM (Fig. 8C'). However, image analysis showed no significant difference for the percentage of neurons in culture and neurite outgrowth of the exposed cells when compared with control (Fig. 9B and D, 10 B and D).

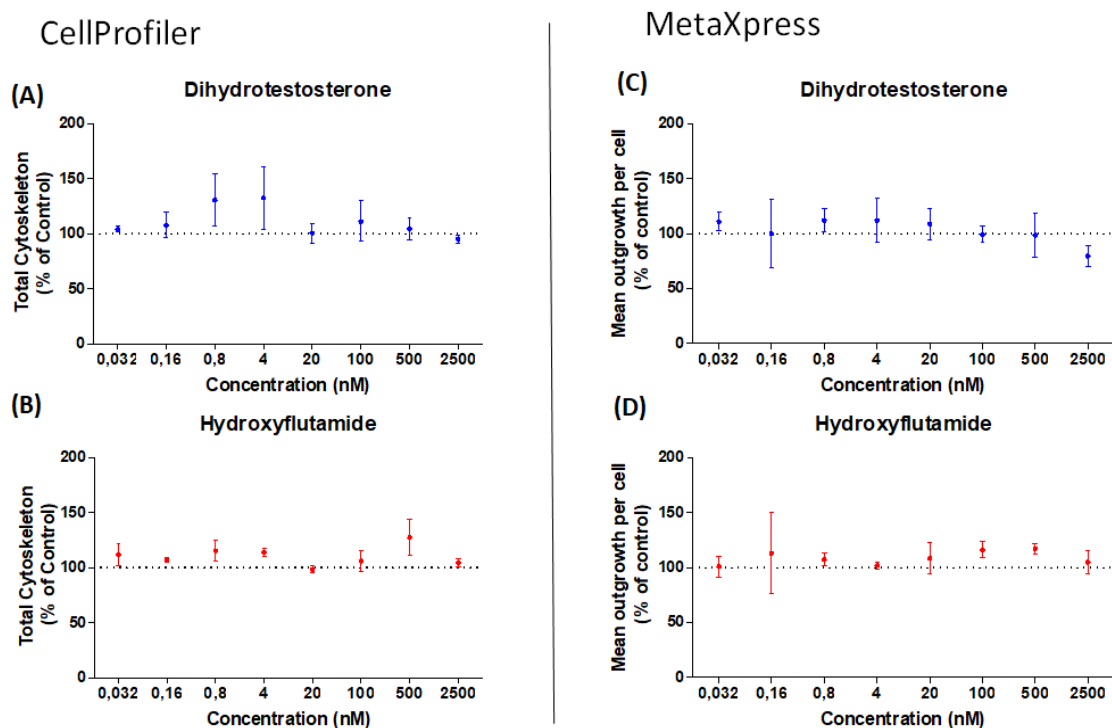


**Figure 8: Morphological changes in C17.2 cells after treatment with AR agonist and antagonist.** After 10 days of differentiation and exposure, the cells were immuno-stained with  $\beta$  III-tubulin antibody (yellow) for neurons, and the nuclei were stained with Hoechst 33342 staining (blue) (A) 0.1% DMSO-negative control (B) Rotenone – positive control (125 nM) (C-F) 0.16, 4, 100, 500 nM dihydrotestosterone (C' - F') 0.16, 4, 100, 2500 nM Hydroxyflutamide.



**Figure 9. Percentage neurons in culture of the C17.2 cell-line on exposure to AR agonist and antagonist.** The Data generated from image analysis with CellProfiler and MetaXpress are presented as a percentage of negative control (cells exposed to 0.1% DMSO). The cells were exposed to agonist (blue dot) and antagonist (red dot) of the receptor. (A) Dihydrotestosterone on percentage neurons from CellProfiler (B) Hydroxyflutamide effect on percentage neurons from CellProfiler (C) Dihydrotestosterone effect on percentage neurons from MetaXpress (D) Hydroxyflutamide effect on percentage neurons from MetaXpress. Data are presented as the mean of three independent experiments performed in triplicates. Bars represent mean  $\pm$  SD. Data was analysed using two-way ANOVA with Bonferroni post-test.





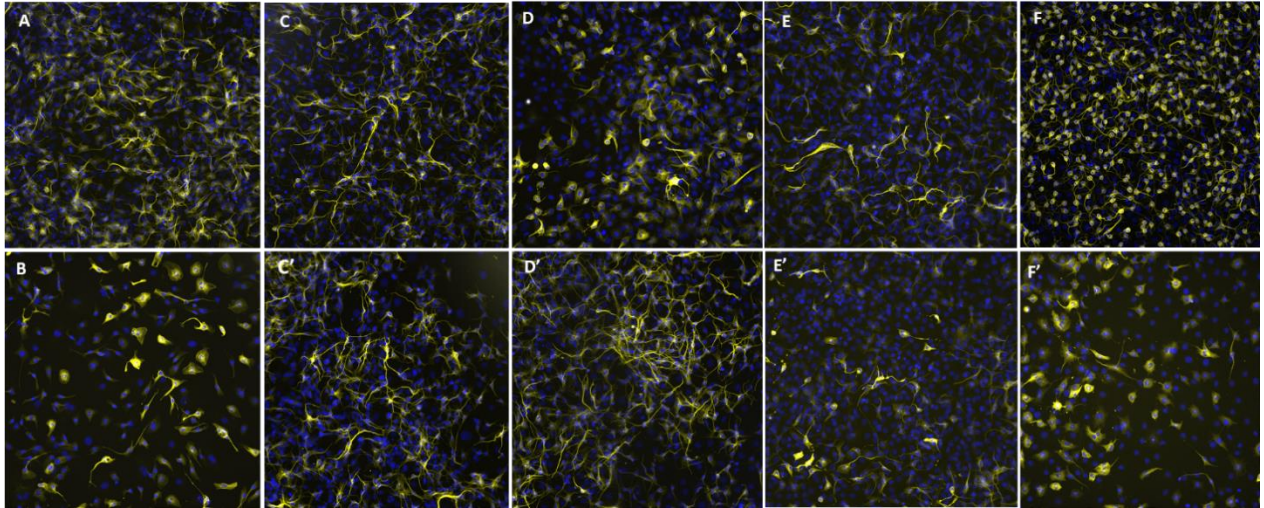
**Figure 10. Neurite outgrowth of the C17.2 cell-line on exposure to AR agonist and antagonist.** The Data generated from image analysis with CellProfiler and MetaXpress are presented as a percentage of negative control (cells exposed to 0.1% DMSO). The cells were exposed to agonist (blue dot) and antagonist (red dot) of the receptor. (A) Dihydrotestosterone effect on total cytoskeleton from CellProfiler (B) Hydroxyflutamide effect on total cytoskeleton from CellProfiler (C) Dihydrotestosterone effect on mean outgrowth per cell from MetaXpress (D) Hydroxyflutamide effect on mean outgrowth per cell from MetaXpress. Data are presented as the mean of three independent experiments performed in triplicates. Bars represent mean  $\pm$  SD. Data was analysed using two-way ANOVA with Bonferroni post-test.

### 3.2.3. Effects of TR agonists/antagonists on morphological neural differentiation endpoints

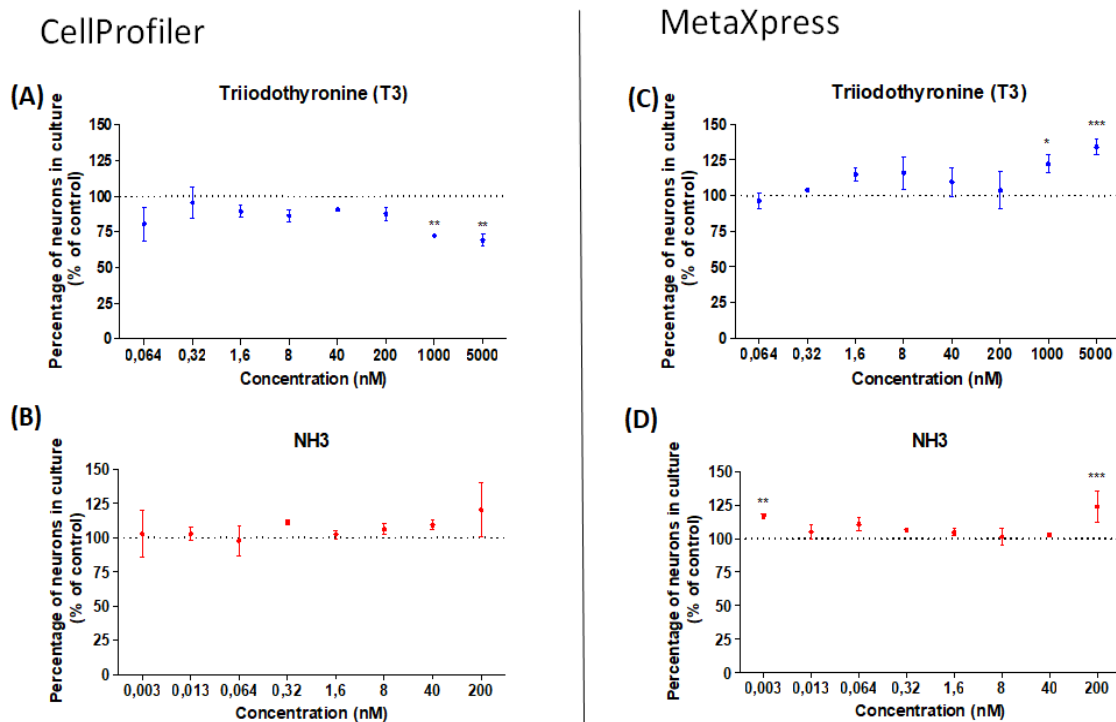
When it comes to TR, visual inspection showed the receptor agonist (triiodothyronine) decreases neuronal cell proportion and induce thinning of neurite outgrowth with increase in concentration (Fig. 11C-F). At the highest concentration tested (5000 nM; Fig. 9F), neuronal cells, with a denser cell body and thin neurite outgrowth were observed. Image analysis with CellProfiler (Fig. 12A) showed significant ( $p=0.01$ ) decrease in percentage of neurons in culture at 1000 nM and 5000 nM, while the MetaXpress (Fig. 12C) showed an increase compared with control. No significant difference in neurite outgrowth of the exposed cells when compared with control for CellProfiler data output (Fig. 13A). However, analysis with MetaXpress depicts neurite outgrowth was significantly ( $p=0.05$ ) higher at 40 nM and 200 nM compared with control.

For the antagonist (NH-3), an increase in the proportion of cell and neuronal cells with increase in concentration was observed (Fig. 11C'-F'). Image analysis of the cells with CellProfiler (Fig. 12B) showed a no significant changes in the percentage neurons in culture, while MetaXpress (Fig. 12D) showed significant ( $p=0.01$ ,  $0.001$  respectively) increase in percentage neurons in culture with at 0.003 nM and 200 nM. For neurite

outgrowth, CellProfiler analysis (Fig. 13B) indicate no significant difference in cell response, while MetaXpress showed significant ( $p=0.05$ ) increase in neurite outgrowth by the cells at 40nM (Fig. 13D).

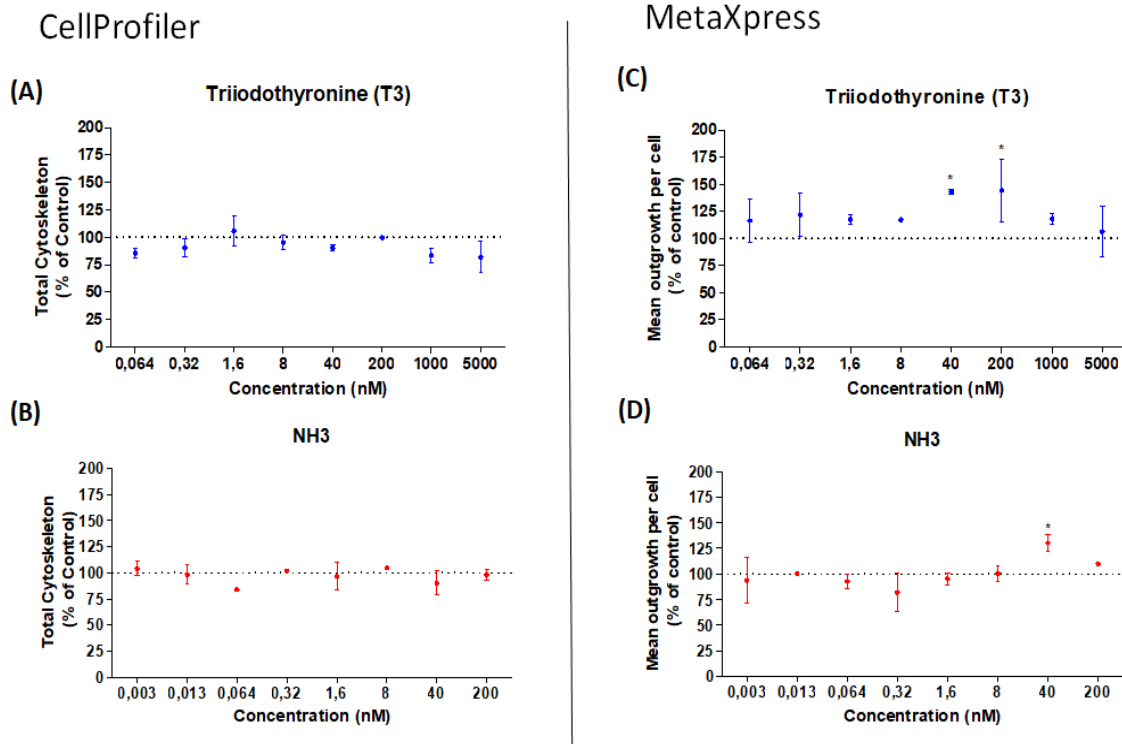


**Figure 11: Morphological changes in C17.2 cells after treatment with TR agonist and antagonist.** After 10 days of differentiation and exposure, the cells were immuno-stained with  $\beta$  III-tubulin antibody (yellow) for neurons, and the nuclei were stained with Hoechst 33342 staining (blue) 0.1% DMSO-negative control (B) Rotenone – positive control (125 nM) (C-F) 0.064, 8, 200, 5000 nM triiodothyronine (C' - F') 0.0128, 0.32, 40, 200 nM NH-3.



**Figure 12. Percentage neurons in culture of the C17.2 cell-line on exposure to TR agonist and antagonist.** The Data generated from image analysis with CellProfiler and MetaXpress are presented as a percentage of negative control (cells exposed to 0.1% DMSO). The cells were exposed to agonist (blue dot) and antagonist (red dot) of the receptor. (A) triiodothyronine on percentage neurons from CellProfiler (B)

NH-3 effect on percentage neurons from CellProfiler (C) triiodothyronine effect on percentage neurons from MetaXpress (D) NH-3 effect on percentage neurons from MetaXpress. Data are presented as the mean of three independent experiments performed in triplicates. Bars represent mean  $\pm$  SD. Data was analysed using two-way ANOVA with Bonferroni post-test  $*p \leq 0.05$ ,  $**p \leq 0.01$ ,  $***p \leq 0.001$ .



**Figure 13. Neurite outgrowth of the C17.2 cell-line on exposure to TR agonist and antagonist.** The Data generated from image analysis with CellProfiler and MetaXpress are presented as a percentage of negative control (cells exposed to 0.1% DMSO). The cells were exposed to agonist (blue dot) and antagonist (red dot) of the receptor. (A) Triiodothyronine effect on total cytoskeleton from CellProfiler (B) NH-3 effect on total cytoskeleton from CellProfiler (C) Triiodothyronine effect on mean outgrowth per cell from MetaXpress (D) NH-3 effect on mean outgrowth per cell from MetaXpress. Data are presented as the mean of three independent experiments performed in triplicates. Bars represent mean  $\pm$  SD. Data was analysed using two-way ANOVA with Bonferroni post-test,  $*p \leq 0.05$ .

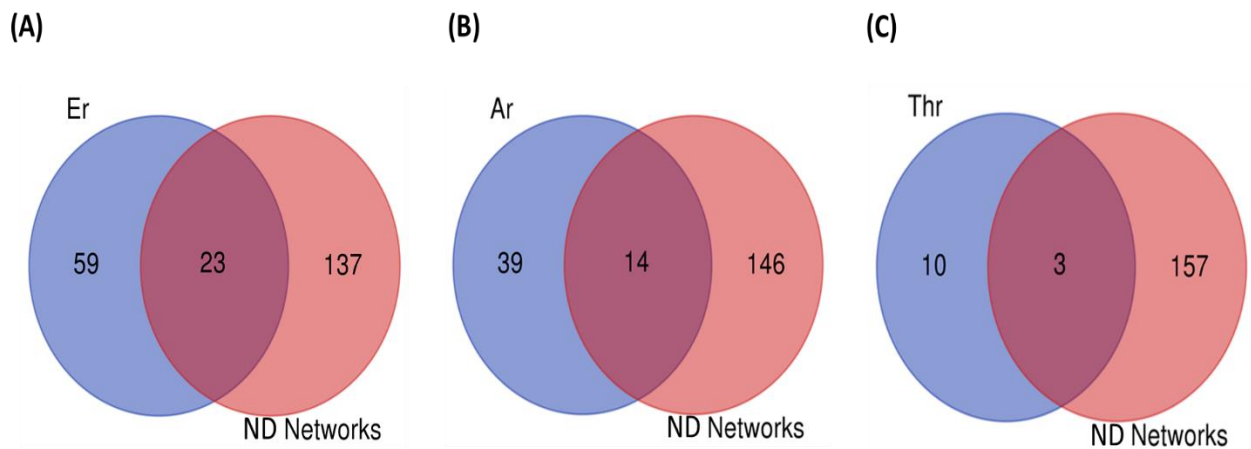
### 3.3. Effects of hormone receptor agonists and antagonists on gene expression

#### 3.3.1. Identification of potential hormone-dependent marker genes for neural development

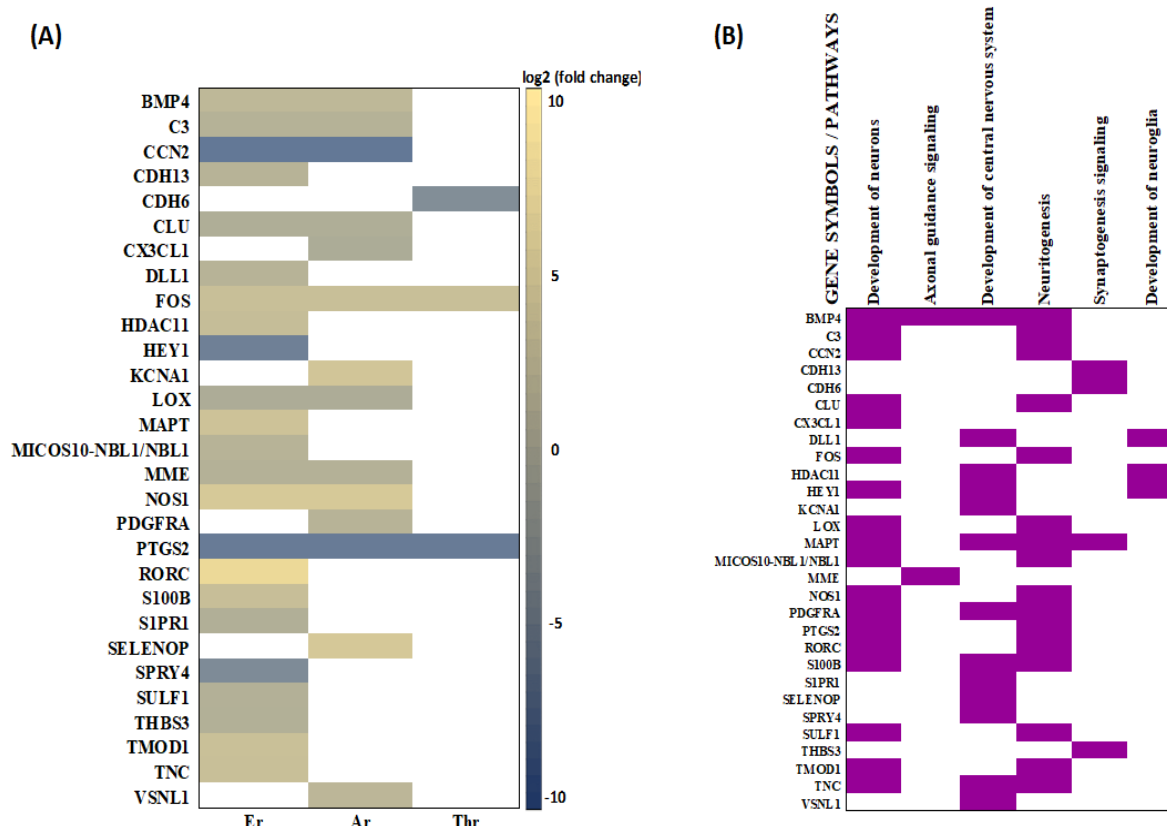
This section focuses on identifying genes regulated by each of the hormonal receptors, necessary for neurodevelopment in the C17.2 cells, that could be use as marker genes for ECD induce DNT in the cell. The Ingenuity Pathway Analysis (IPA) software was used to run canonical pathway analysis on the C17.2 cell-line 7 days RNA-seq data (Gluga et al., 2017) and 10 days microarray data (Attoff et al., 2017), at  $p < 0.00005$  and fold change threshold equal or higher than  $\pm 2$ ,  $\pm 1.75$  respectively. Six networks, comprising development of neurons, development of neuroglia, development of central nervous system, neuritogenesis, axonal guidance signalling, and synaptogenesis signalling pathway

were significantly associated with ND, and 160 associated DEGs were mapped out for these pathways.

For downstream analysis of targeted genes, regulated by AR, ER, and TR from the two C17.2 cell transcriptomic data, genes with p-value < 0.00005 and fold change threshold equal or higher than  $\pm 2$ ,  $\pm 1.75$  for RNA-seq and microarray data respectively, were defined as differentially expressed. In this analysis, only oestrogen, oestrogen receptor 1 and 2 (ESR1 and ESR2), dihydrotestosterone, AR, and thyroid hormone receptor beta (THRB) gave predictable target genes for the three hormonal pathways studied from the C17.2 transcriptomic data, while thyroid hormone receptor alpha (THRA) and T3 did not give predictable target genes. A total of 98, 183 and 20 target genes regulated by AR, ER, and TR, respectively was identified. Venn diagrams were then used to identify DEGs involved in neurodevelopment and linked to the different hormonal pathways in the C17.2 cells (Fig. 14). This analysis identified 14, 23, and 3 DEGs linked to AR, ER, and TR regulation of neurodevelopment, respectively. These genes are presented in the heat map for the different receptors and pathways (Fig. 15 A-B). As such, a total of 29 DEGs were identified as potential marker genes involved in ND, being regulated by the AR, ER and TR pathways. Among the 29 identified DEGs, FOS and PTGS2 were the common gene linked to all three pathways of ND.



**Figure 14. Venn diagram showing overlap of differentially expressed genes between the hormonal receptors and ND networks.** A) for Oestrogen receptor, B) for Androgen receptor, and C) for thyroid receptor. Blue circle-hormonal receptor downstream genes in C17.2 cell. Red circle-ND genes in C17.2 cell. Blue and red circle overlap-hormonal receptor linked ND genes in C17.2 cell.

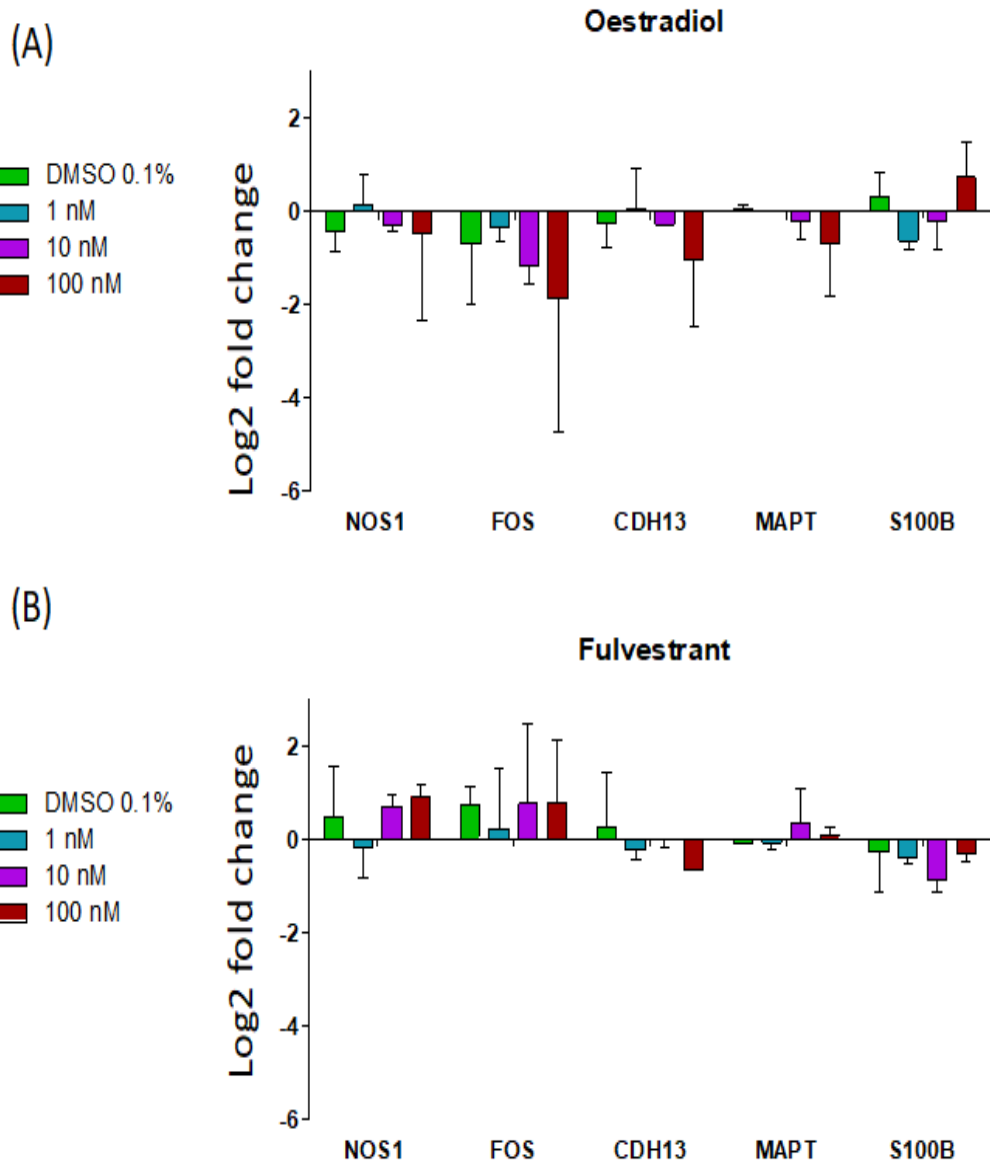


**Figure 15. Heat map of the selected genes for neural development via hormonal regulation in the C17.2 cell-line.** 29 of the ND genes could be linked to the AR, ER, and TR pathways. A) Heat map of the 29 selected genes, according to their log2 (fold change) for the AR, ER, and TR pathways B) A map showing the pathways/networks the selected genes are involved in as identified from IPA database.

### 3.3.2. Validation of hormone-dependent marker genes for neural development

In order to validate the expression, specificity and sensitivity of the identified hormonal linked neurodevelopmental DEGs of C17.2 cells, as marker genes, RT-qPCR technique was applied. Using primer pairs designed for each of the genes,  $\beta$ -III tubulin (a neuron marker), and two selected reference genes (Tbp, Rplp1), their efficiency was tested with RT-qPCR. In total, 21 of these genes, with efficiency ranging from 0.8 to 1.1 worked, and are presented in appendix 2.

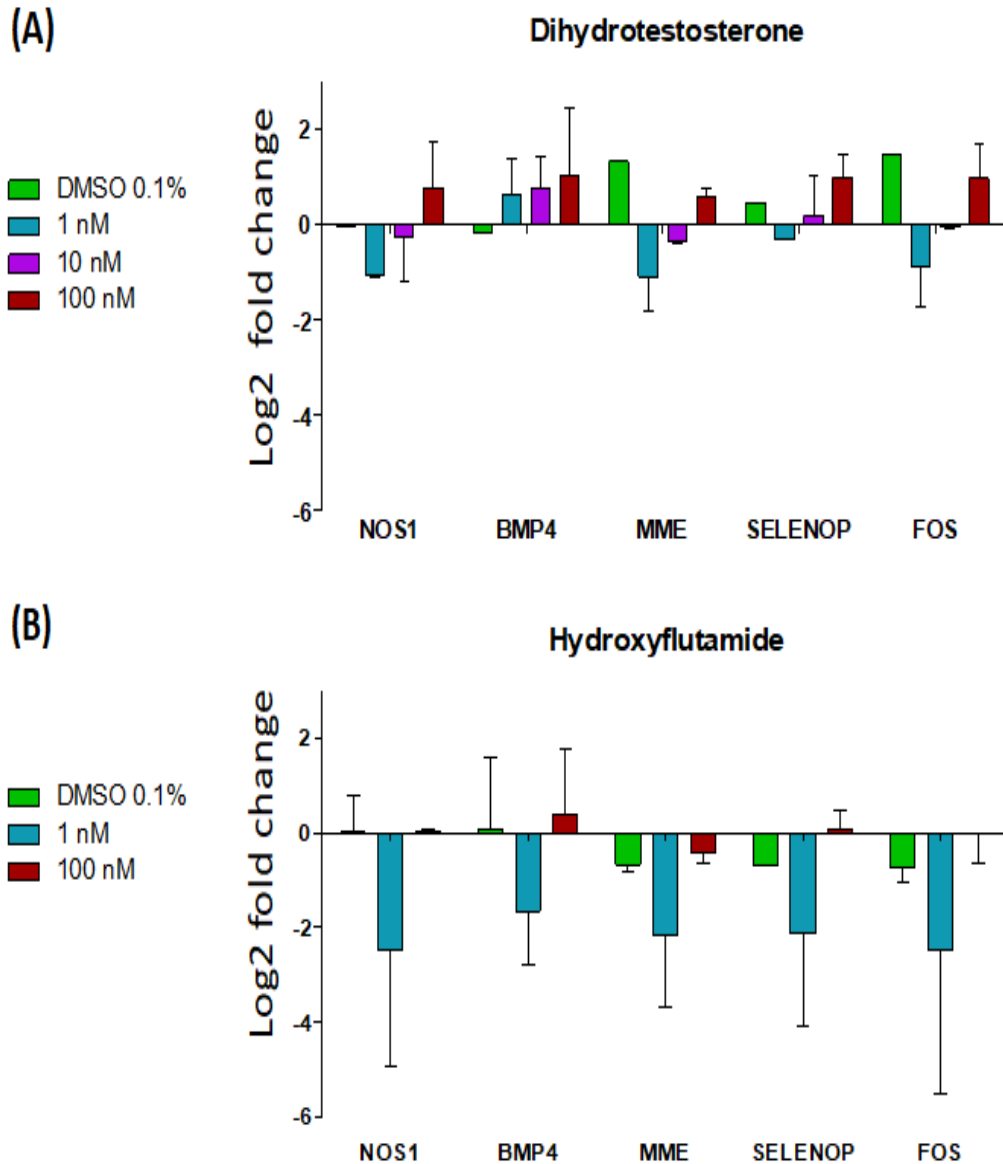
To ascertain that the selected DEGs are marker genes for hormonal induced effects on neural differentiation, the expression of some of these genes were studied following treatment with the hormonal agonist and antagonist. To do this, the non-cytotoxic concentrations of 100nM, 10nM and 1nM were used, based on the IC10 from viability assay, morphological changes and documented effective concentrations of each agonist and antagonists. Unfortunately, due to time constraints, experiments with ER and AR target genes could only be run in biological duplicates, while for TR only one replicate was run. Thus, results presented here can give indications of effects but will have to be confirmed in future experiments. For ER, the differential expression of five marker genes, NOS1, FOS, CDH13, MAPT and S100B in response to the agonist and antagonist treatment indicate that oestradiol (Fig. 16A) tends to downregulate the gene expression, while for fulvestrant NOS1 and FOS were upregulated (Fig. 16B).



**Figure 16: Differential expression of marker genes in the C17.2 cell in response to ER agonist and antagonist treatment.** A) oestradiol B) Fulvestrant. Cells were treated with 1 nM, 10nM, 100nM each of oestradiol and fulvestrant. 0.1% DMSO was used as negative control. Data are presented as the mean of two independent experiments performed in triplicates. Bars represent mean  $\pm$  SD.

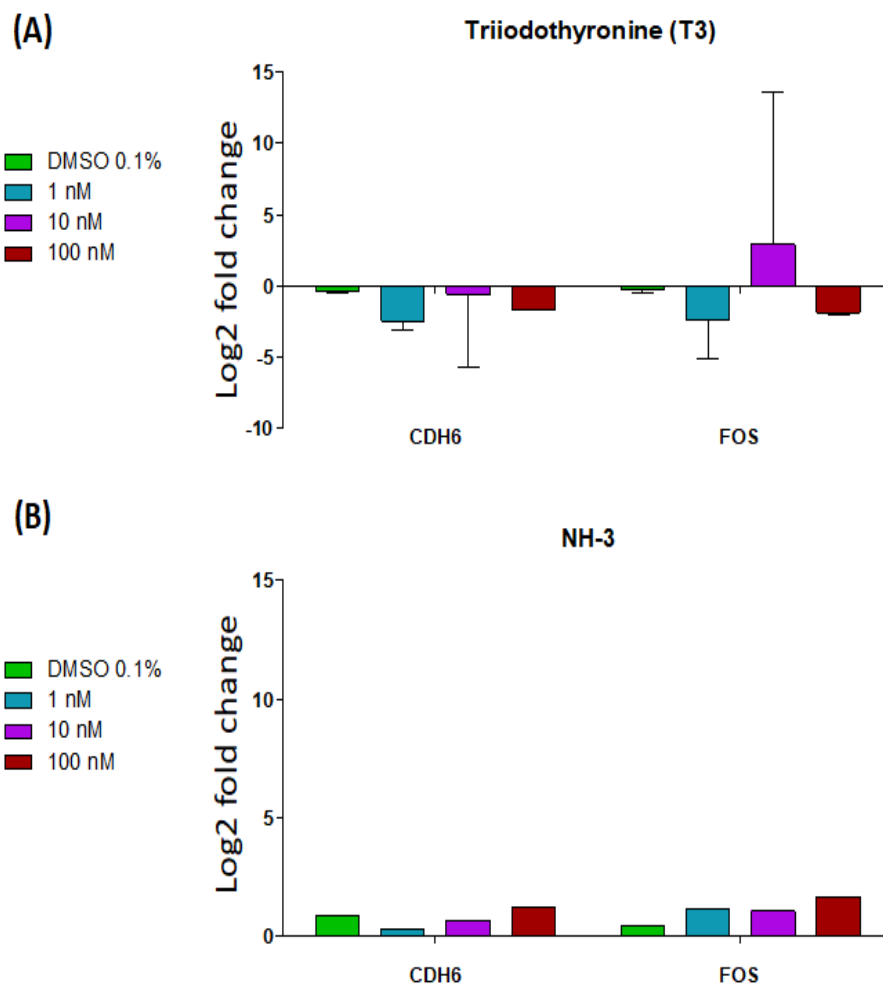
For AR, the expression of five marker genes, NOS1, BMP4, MME, SELENOP and FOS in response to the agonist and antagonist treatment indicates that the agonist tends to upregulate the expression of these genes, while the antagonist tends to downregulate the expression of these genes (Fig. 17).





**Figure 17: Differential expression of marker genes in the C17.2 cell in response to AR agonist and antagonist treatment.** A) Dihydrotestosterone B) Hydroxyflutamide. Cells were treated with 1 nM, 10nM, 100nM each of dihydrotestosterone and hydroxyflutamide. 0.1% DMSO was use as negative control. Data are presented as the mean of two independent experiments, or one independent experiment performed in triplicates. Bars represent mean  $\pm$  SD.

In the case of TR, the expression of two marker gene, CDH6 and FOS, in response to the agonist and antagonist treatment indicates that triiodothyronine (Fig. 18A) tends to downregulate the expression of this genes, while NH-3 upregulated them (Fig. 18B).



**Figure 18: Differential expression of marker genes in the C17.2 cell in response to TR agonist and antagonist treatment.** A) Triiodothyronine B) NH-3. Cells were treated with 1 nM, 10nM, 100nM each of triiodothyronine and NH-3. 0.1% DMSO was use as negative control. Data are presented as the mean of two independent experiments, or one independent experiment performed in triplicates. Bars represent mean  $\pm$  SD.

## 4.0 DISCUSSION

The endocrine system plays an important role in proper development of vertebrate organisms, especially in brain development. However, this system is prone to toxic insults from chemicals, so called EDCs, resulting in possible developmental and functional defects in exposed organisms and/or its progeny. One such effect is neurodevelopmental defects from prenatal and perinatal exposure in the foetus. Neurodevelopmental disorders such as intellectual disability (ID), autism spectrum disorders (ASDs), attention deficit (hyperactivity) disorder (ADHD) and schizophrenia share similar cytoarchitectural, connectional and functional features which indicates a similar origin (Schubert et al., 2015). They have complex aetiology that involve many genes and environmental factors (Schubert et al., 2015). However, detailed molecular and mechanism underlining these disorders are still unclear. EDCs could induce these disorders by disrupting hormone



receptor signalling (Street et al., 2018; Thomas Zoeller et al., 2012), leading to alteration in the expression of key genes regulating brain development.

The increase in neurodevelopmental disorders diagnosis in children in recent times, has led to the need for development of methods to evaluate DNT induced by exposure to chemicals (Attoff et al., 2017; Herbert, 2010). However, the current guidelines for DNT testing uses *in-vivo* models with endpoints such as behaviour, brain weight and neuropathology (OECD 2007; US EPA 1998). As such, there is a need to develop rapid, high-throughput *in-vitro* methods, applicable for the screening of DNT effects of chemicals (Attoff et al., 2017; Council, 2007; Fritsche et al., 2017). Herein, the murine neural progenitor cell line C17.2, was used as a model for *in vitro* hormonal linked DNT screening. The C17.2 cell has been shown to have similar neurotoxicological response to those observed *in-vivo*, due to its ability to differentiate into neurons and astrocytes, as astrocytes are known to modulate neuron sensitivity to chemicals (Lundqvist et al. 2013). In recent times, C17.2 cells have been used to study DNT inducing chemicals like methylmercury, manganese, acrylamide, and arsenic (Attoff et al., 2016, 2017; Rocha et al. 2011; Tamm et al., 2006, 2008).

To investigate hormonal linked DNT in the C17.2 cell-line, a non-cytotoxic but effective concentration of the hormones need to be established, as a rule for assessing adverse events in organ type test systems (Attoff et al., 2017; Leist et al., 2010). To this end cytotoxicity and viability assay was carried out using varied concentrations of the receptor's agonists and antagonists. The cytotoxicity data showed cytotoxicity induced only for AR agonist/antagonist at some of the tested concentrations relative to control. This response could be due to the nature of the assay used. The assay measures the level of lactate dehydrogenase (LDH) released by the dead cell. In principle, the half-life of LDH is 9 hours. Given this, and the fact that this experimental design runs for 10-day, it is possible that the LDH released by the cells, in response to the agonists and antagonists, has degraded to some extent before the assay was carried out, thus affecting the data output. Additionally, the standard deviation of the data generated from this assay were large, and this give some level of uncertainty about the assay. As such, no cytotoxicity was detected for the cells at the tested agonist and antagonist concentrations except for AR agonist/antagonist.

The viability assay is used to measure cell metabolic activity in viable cells. An inhibitory concentration IC<sub>10</sub>, which is considered as noncytotoxic and used to study adverse events was estimated for each agonist and antagonist with this assay (Attoff et al., 2017; Krug et al., 2013; Rempel et al., 2015). When it comes to the cell response to the oestrogen receptor agonists and antagonists, no difference was observed for the metabolic activity of the exposed cells relative to the control. However, the highest concentration of 2.5µM and 0.5µM for oestradiol and fulvestrant respectively, having reduced metabolic activity, were not considered for the marker gene validation as they were toxic to the cells. In addition, the estimated IC<sub>10</sub> for oestradiol was 0.25µM. As such, this concentration range of oestradiol and fulvestrant were considered for the validation of ER linked marker genes of DNT. Further, the cells metabolic activity in response to the AR hormones and TR agonist (triiodothyronine) treatment were similar to the control. Thus, the tested concentrations can be considered for the validation of the DNT marker genes. On the other hand, the cell metabolic response to NH-3 (TR antagonist) was significantly low at 5µM and 1µM when compared with the control cells. This implies the two concentrations of NH-3 were toxic

to the cells. The estimated IC<sub>10</sub> for NH-3 was 0.53μM and was considered for the validation of TR linked marker genes for DNT. The cell viability assay tends to be a more sensitive assay, suitable for this experimental design, compared with the cytotoxicity assay, because it deals with metabolically active cells which is related to the total number of viable cells in the culture after exposure to agonist/antagonist. As such, it can be run after the exposure duration, without risk of degradation of target agent, thus making it a better fit for these 10-days experimental design.

Further in the study, after establishing the non-cytotoxic concentration of each agonist/antagonist, the effectiveness of these receptor agonist/antagonist in inducing morphological alteration in the C17.2 cell line, was examined. This was carried out using morphological endpoints such as total cell count, ratio of neurons and neurite outgrowth upon staining of the cells with β III-tubulin (neuron marker) and Hoechst (nuclei marker), after treatment with the different agonist/antagonist. These endpoints have been used previously to study DNT effect of methylmercury, acrylamide and valproic acid, which are known neurotoxic substances on the C17.2 cell line (Attof et al. 2017). They reported that the number of neurites per neuron (total neurite process) were reduced by these substances, with valproic acid having reduced number of neuronal cells and neurites the most. This depicts the C17.2 cell as a sensitive model for examination of functional defects, induced by toxic chemicals on the brain.

The C17.2 cell originates from the brain cerebellum, which has less cellular heterogeneity than the other part of the brain (Chatonnet et al. 2012). It was stated that cellular heterogeneity of the brain cell masks variation in gene expression of a cell type (Chatonnet et al. 2012), and this could impair the identification and expression of hormonal receptor target genes in a study like this. As such, optimal culture conditions of this cellular model favouring neurons or glial cells is of necessity. Image analysis using CellProfiler and MetaXpress depicts a 30% and 16% neuron ratio to cells estimate in culture, while manual counting generated a 9% neuronal ratio to cells. This disparity could be due to underestimation from manual counting, due to clustering of the cell. Also, the removal of images which are out of focus for CellProfiler analysis could be a reason for the deviation from MetaXpress result. An overestimation from the CellProfiler software is also possible from the detection limit of the pipeline used. However, this result is similar to the report by Attof et al. (2017) following the differentiation of C17.2 cell-line, in serum-free N2 medium with growth factors NGF and BDNF, indicating 20% and 35% neuronal cells, after 5- and 10-days differentiation period, by counting cells in phase contrast images. In my study, no growth factor was used for the cell culture compared with Attoff et al. (2017), thus suggesting the cells were growing well.

Image analysis with CellProfiler and MetaXpress indicates no difference in percentage neuron in culture and neurite outgrowth at the non-cytotoxic concentration of oestradiol. In the case of fulvestrant, both analyses showed no significant difference in neurite outgrowth, however MetaXpress indicates increase in percentage neuron with increase in concentration. This thus suggest fulvestrant does not elicit effect on neurite outgrowth but induce the C17.2 cell potential to differentiate into neuron. Unlike in my study, Fujiwara et al. (2018), using a ReNcell VM which is a human fetus-derived neural progenitor cell line, showed that oestrogen ( $10^{-10}$ M) and bisphenol A (an ER agonist;  $10^{-16}$  and  $10^{-10}$  M)

suppresses neural differentiation. A possible reason why Fujiwara et al. (2018) observed response to oestrogen treatment at  $10^{-10}$ M could be because of the cell type used for the study, which is a human neural progenitor cell-line derived from ventral mesencephalon region of a developing brain, while the cell-line used here is from a 4-day old postnatal mouse cerebellum. These are from different region of the brain and different animal species, with potential to elicit different response to hormonal treatment. Some other studies have also shown BPA exposure suppresses synapse formation, disrupts neural migration, increases the number of glial cells, and upregulates neural cytoskeletal proteins (Fujiwara et al., 2018; Iwakura et al., 2010; Komada et al., 2012; Leranthe et al., 2008; Nakamura et al., 2007; Yamaguchi et al., 2006; Yokosuka et al., 2008).

Androgen hormones are known for their integral role in sexual differentiation of the brain and regulation of genes involved in neurotransmitter production and release, synapse conformation changes, control of neuronal apoptosis and alteration of neurochemical profiles (Alonso-Solís et al., 1996, Kelemenova & Ostatnikova, 2008). Herein, exposure of C17.2 cells to dihydrotestosterone and hydroxyflutamide has no effect on neurite outgrowth and percentage neuron in culture in the cell-line. As such AR is not involved in DNT using these endpoints.

Thyroid hormones and its receptors play pivotal role in brain development. They are important for proper neuronal and glial differentiation, neuronal migration, and myelination. Thyroid hormone deficiency in human brain development has been associated with irreversible mental retardation and variable degrees of neurological impairment (Chatonnet et al. 2015). When it comes to TR, triiodothyronine was observed to decrease proportion of neuron to cell in culture. The reduction in neuron ratio to cell was more pronounced at  $1\mu\text{M}$ . Although this concentration is not endogenously relevant, but it was not cytotoxic considering the viability assay. Image analysis with CellProfiler indicates significant decrease in percentage neuron in culture at  $1\mu\text{M}$  and  $5\mu\text{M}$ , while the MetaXpress depicts increase in percentage neuron at similar concentration. Even though both analyses gave different response trend, they were able to show the cell were responding to T3 treatment. Only MetaXpress showed changes in neurite outgrowth at 40 nM and 200 nM compared with control which agrees with what was observed. The TR antagonist, NH-3, was observed to increase neuron ratio to cell with increase in concentration except at 40 nM. It also impaired neurite outgrowth at 40-200nM. No changes in the percentage neurons in culture was seen with CellProfiler analysis, while MetaXpress showed increase in percentage neurons in culture at 0.003 nM and 200 nM. For neurite outgrowth, MetaXpress showed increase in neurite outgrowth by the cells at 40nM, while no response was detected with CellProfiler analysis. This thus suggest the important role of thyroid hormone in neuronal cell proliferation, differentiation, and induction of neurite outgrowth at physiological concentrations.

The proper wiring of the brain depends on neurite outgrowth during development (Stiegler et al., 2011). Effect on neurite outgrowth could be associated with some defect in the neuronal cytoskeleton (Fujiwara et al. 2018). The microtubule-associated protein 2 (MAP2), a cytoskeleton-related protein in neurons, whose expression has been associated with schizophrenia (Fujiwara et al., 2018; Golub et al., 2010) could be attributed to effect on neurite outgrowth and may cause abnormal brain development and behavioural

alterations. This neurite connections are affected in individuals with schizophrenia, anxiety disorders, or reduce IQ and axonal pruning can also be self-destructive in the developing neurons (Saxena & Caroni, 2007). Another pathway that can affect neurite outgrowth by harmful effects of toxicant is the mitogenic-activated protein kinases (MAPK), protein kinase C (PKC) and GTPase rho signalling (Chen et al., 2000; Doherty et al., 2000; Larsson, 2006) which are needed for functional and uncompromised neurite outgrowth.

Overall, based on the imaging analysis, the C17.2 cell did not show any morphological response to ER and AR agonist/antagonist at the noncytotoxic concentration tested. TR agonist/antagonist on the other hand elicited effect on the cell neurite outgrowth and percentage neuron in culture at 40 and 200 nM. Of the two-imaging analysis used, MetaXpress software seem to be more sensitive than CellProfiler in detecting the cell response. This is because the MetaXpress software is built for neurite analysis, and it is more sensitive to analyse images even at low resolution. Also, the two software were unable to assess some important parameters such as impairment and thinning of the neurite outgrowth.

In recent times, toxicogenomics, using transcriptomic studies, has become a vital part of toxicology (Attoff et al., 2017; Chen et al., 2012). The approach examines changes in gene expression, from exposure to xenobiotics, thus giving insight to the toxicity mechanism of xenobiotics. This approach is important in DNT, for the identification of chemicals, altering key gene expression during neuronal differentiation and development (Attoff et al., 2017). However, transcriptomic approaches generate enormous amounts of data and yet using a small set of predetermined biomarkers will not help in identifying all DNT inducing chemicals. Thus, *in-silico* prediction of possible target genes of chemicals is being used to identify potential marker genes from the transcriptomic data. This approach has shown useful for the selection of relevant biomarkers (Attoff et al., 2017). In this study, *in-silico* analysis of transcriptomic data was then used to identify potential marker genes, driving hormonal linked ND responses in the C17.2 cell-line. In total, 29 marker genes regulated by the AR, ER and TR pathways, which are involved in neuronal differentiation of the C17.2 cell (via the neurogenesis, development of neuroglia, development of central nervous system, neuritogenesis, axonal guidance signaling, and synaptogenesis network) were identified. In consistency with this data, five of these genes (BMP4, S100B, SIPRI, MAPT and CX3CLI), were among the 30 genes reported by Attoff et al. (2017) to be involved in neural differentiation of the C17.2 cell. Among the 29 identified DEGs, FOS and PTGS2 were the common genes associated with the three hormonal pathways understudied for ND. Suggesting their importance in hormonal regulation of brain development.

Interestingly, while analysing the targeted genes regulated by AR, ER, and TR from the two C17.2 cell transcriptomic data sets, only oestrogen, oestrogen receptor 1 and 2 (ESR1 and ESR2), dihydrotestosterone, AR, and thyroid hormone receptor beta (THRB) gave predictable target genes for the three hormonal pathways understudied. The absence of thyroid hormone receptor alpha (THRA) responsive genes from the C17.2 transcriptomic data depicts non-responsiveness of the cell line to THRA. This is not far-fetched as a study by Chatonnet et al. (2012) on transcriptomic analysis of TR-alpha target genes involved in cerebellum development made use of TR $\alpha$ 1 transfection of the C17.2 cells, to restore its

response to T3. Of the 15 genes they reported to be T3 responsive in the C17.2/TR $\alpha$ 1 cells, none was found among the TR linked DEGs from this study. Another report from (Chatonnet et al., 2015), gave 28 direct TR regulated genes in the brain and C17.2 cells. The brain data was generated from 10 studies on T3/TR target genes in the brain, while the C17.2 data was from ChIP-Seq data of TR $\alpha$ 1 and TR $\beta$ 1 transfected C17.2 cells. Among the 28 TR regulated genes, only FOS was detected in this study. Since FOS was not identified in Chatonnet et al. (2012) study with C17.2/TR $\alpha$ 1 cells, this suggests it is a TR $\beta$ 1 responsive gene when compared with this study.

It is important to note also that TR gave a low number of target genes (only CDH6, FOS and PTGS2) when compared with the other receptors (ER with 23 DEGs and AR with 14 DEGs). Because of the possibility for long distance transcriptional regulation, it is expected that TR would have many target genes, however this is not so. Chatonnet et al. (2012) transcriptomics studies on TR-alpha target genes in cerebellar cells has also shown a limited number of target genes involved in cerebellum development. They reported that among the 15 T3 responsive genes identified in cerebellar cells, only four (Hr, Dbp, Klf9, Gbp3) were direct TR target genes. As such, other changes in gene expression from T3, could be by variation in neurotrophic factor (Chatonnet et al., 2012; Pogue et al., 2003). It is good to have in mind that the inconsistency and few overlaps in transcriptomic analysis of gene expression important for brain development could be due to the different target gene in the different cell-types, brain area, developmental stages, and experimental conditions, influencing the outcome of this studies.

The expression of some of the identified hormonal linked neurodevelopmental DEGs of C17.2 cells were examined, to ascertain their specificity and sensitivity as marker genes for hormonal induced effects on neural differentiation. This can be considered as a preliminary result because more biological replicates are needed, as such the data need to be interpreted with caution. However, we can show that all the five marker genes examined for ER, (NOS1, FOS, CDH13, MAPT and S100B) were down regulated by oestradiol, while NOS1 and FOS were upregulated by fulvestrant. Indicating, the cells were responding to the ER agonist and antagonist treatment. Also, the expression of five marker genes (NOS1, BMP4, MME, SELENOP and FOS) for AR, by the cell were upregulated by dihydrotestosterone, and down regulated by hydroxyflutamide. Treatment of the cells with TR agonist, downregulate the expression of CDH6 and FOS in the C17.2 cell, while NH-3 upregulated them.

## CONCLUSION

Overall, the imaging analysis with MetaXpress software, *in-silico* prediction and RTq-PCR protocol used are optimized tools for testing developmental neurotoxicity. This C17.2 cell model is a sensitive tool for evaluating morphological and molecular changes at physiological concentrations of the TR agonists or antagonists but not for ER and AR. As such, it cannot be established as sensitive tool for detecting endocrine disrupting induced developmental neurotoxicity via AR or ER. However, the cytotoxicity and morphological changes observed with the TR agonist/antagonist suggest it is a promising model for testing DNT induced by thyroid hormone signalling disruption.

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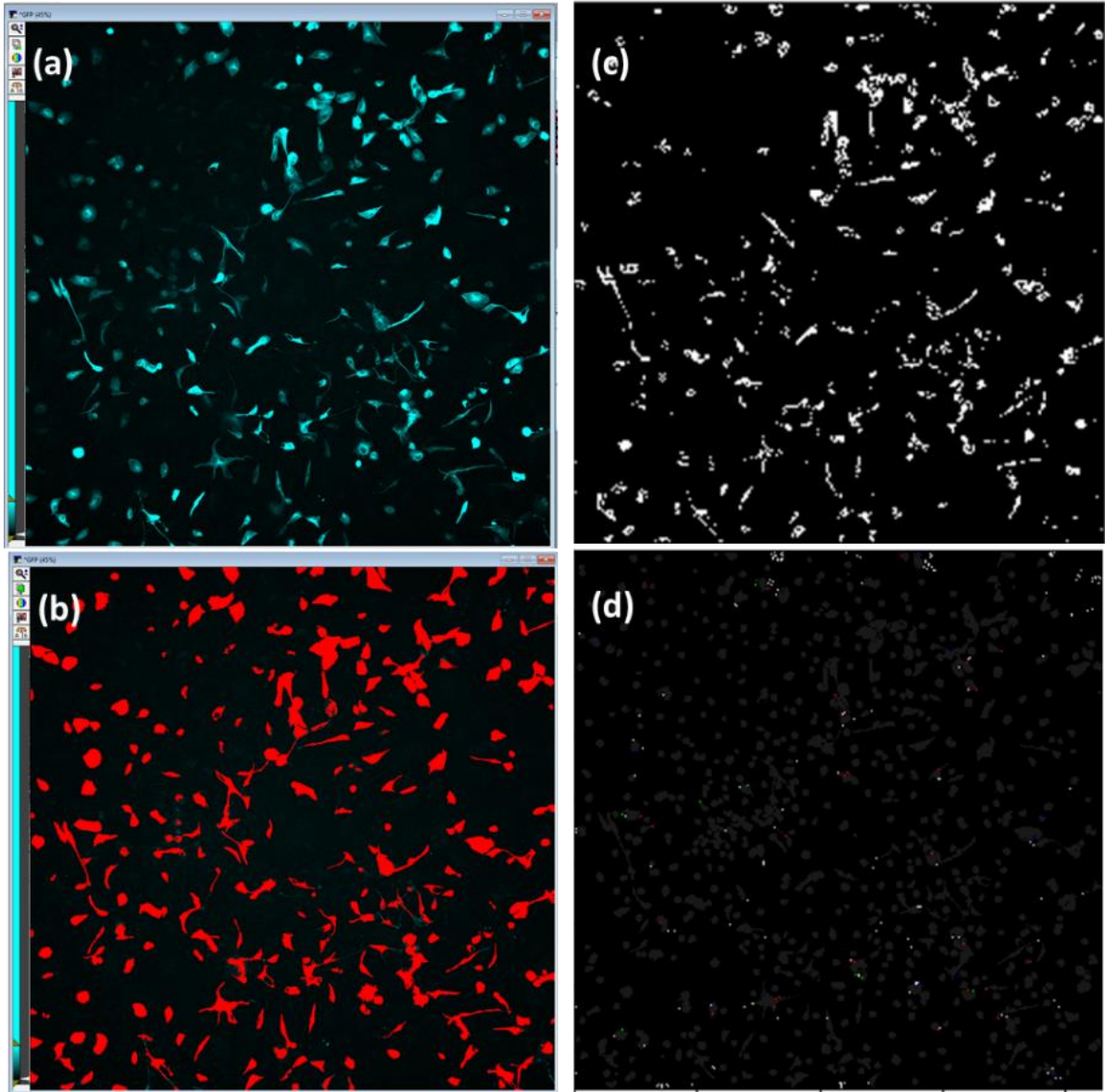
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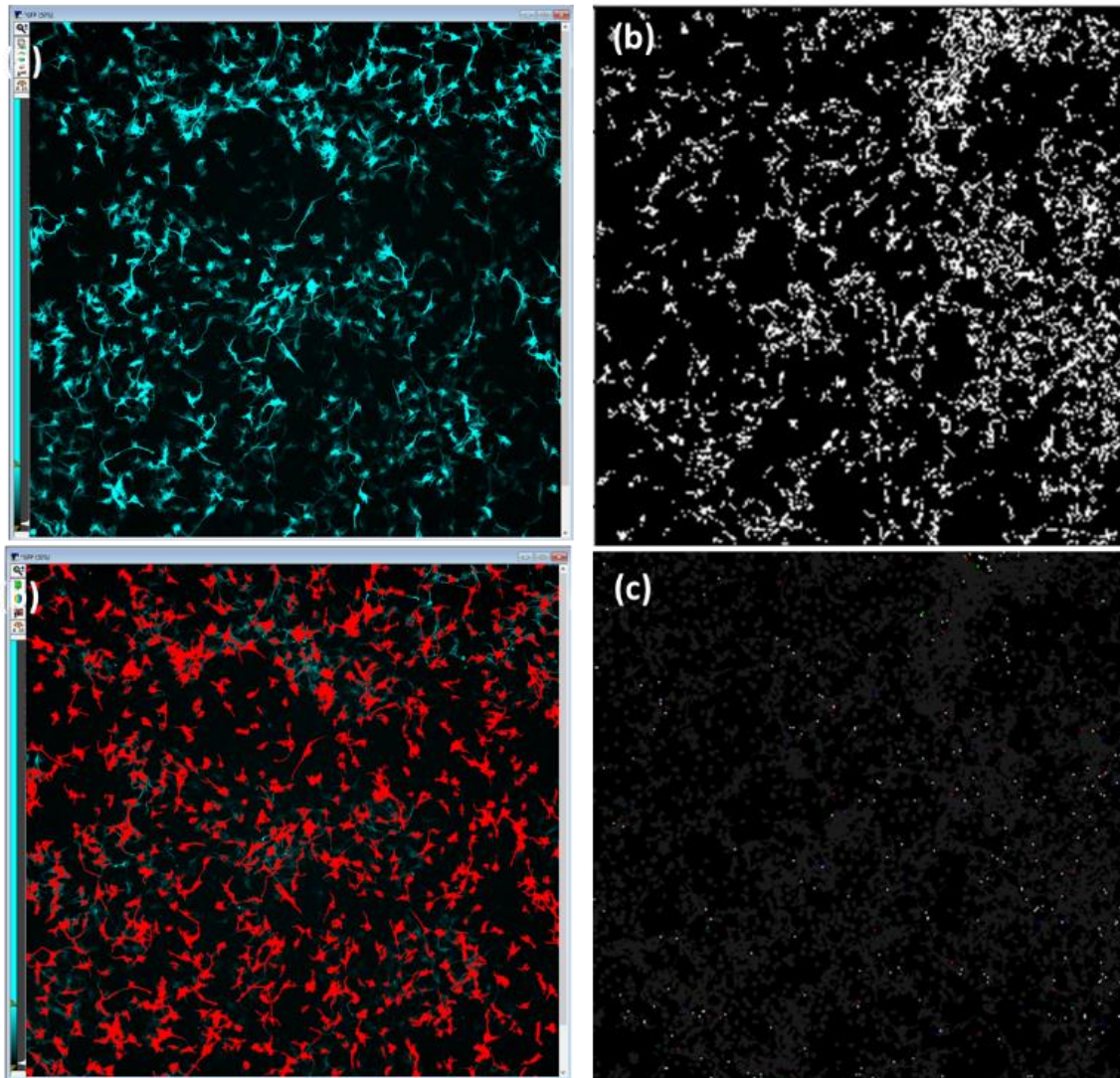
## Appendix 1

### Appendix 1A



**Supplementary Figure 1: Neurite outgrowth analysis with MetaXpress and CellProfiler softwares.** Image of positive control (Rotenone 125 nM) culture cell, immuno-stained with  $\beta$  III-tubulin antibody for neuron. (a) Image of neurons as identified by MetaXpress (b) Image of neurons with as identified by MetaXpress for neurite outgrowth analysis (c) Image of neurons as identified by CellProfiler (d) Image of neurons with as identified by CellProfiler for neurite outgrowth analysis using neurite cytoskeleton.

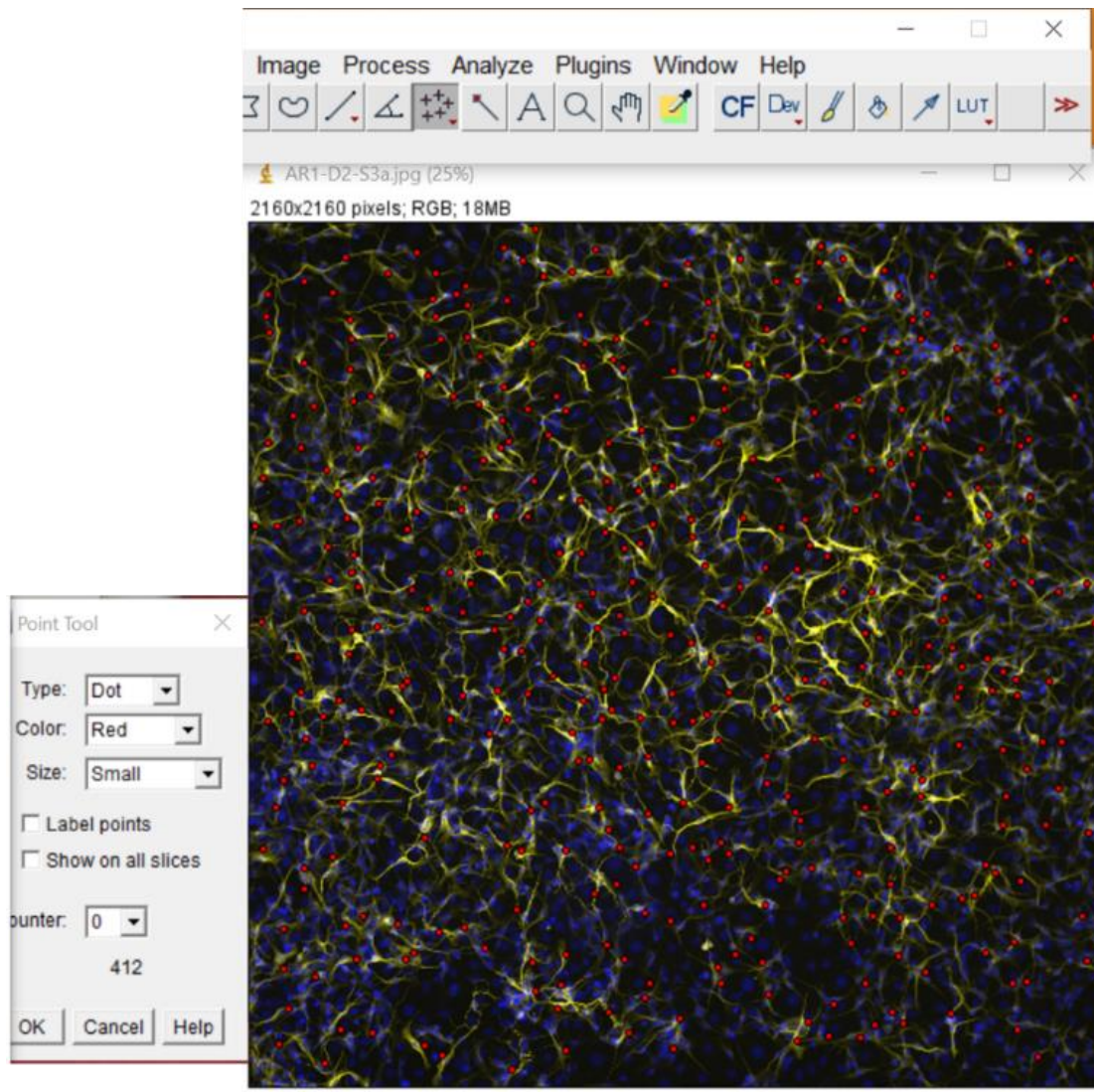
## Appendix 1B



**Supplementary Figure 2: Neurite outgrowth analysis with MetaXpress and CellProfiler softwares.** Image of 0.1 % DMSO-negative control culture cell, immuno-stained with  $\beta$  III-tubulin antibody for neuron. (a) Image of neurons as identified by MetaXpress (b) Image of neurons with as identified by MetaXpress for neurite outgrowth analysis (c) Image of neurons as identified by CellProfiler (d) Image of neurons with as identified by CellProfiler for neurite outgrowth analysis using neurite cytoskeleton.



## Appendix 1C



**Supplementary Figure 3: Manual cell count using ImageJ software.** Image of 0.1 % DMSO-negative control culture cell, immuno-stained with  $\beta$  III-tubulin antibody (yellow) for neurons and the nuclei stained with Hoechst 33342 staining (blue). Red dots stand for counted neurons with the cell counter.

## Appendix 2

**Supplementary Table 1: List of tested and efficient primers of hormonal linked ND marker genes**

Genes	Amplicon size	Forward <sup>a</sup>	Reverse <sup>a</sup>	E
BMP4	118	TTGATACCTGAGACCGGGAAG	ACATCTGTAGAAGTGTGCGCTC	0.86
C3	188	ACTGTGGACAACAACCTACTGC	GCATGTTTCGTAAAAGGCTCGG	0.98
CCN2	222	AGCGGTGAGTCCTTCCAAAAG	TTCCAGTCGGTAGGCAGCTA	0.83
CDH13	181	ATGGCAGAACTCGTGATTGTC	CGCTATCGACTACCTTGCCG	0.98
CDH6	225	GAGAACACAGGCGACATACAG	GACCACAAATGTGCCAACATC	1.06
CLU	164	AGCAGGAGGTCTCTGACAATG	GGCTTCCTCTAAACTGTTGAGC	0.86
CX3CL1	73	GGCTTTGCTCATCCGCTATC	CTGTGTCGTCTCCAGGACAATG	0.95
DLL1	159	GACCTCGCAACAGAAAACCCA	TTCTCCGTAGTAGTGCTCGTC	1.01
FOS	121	ATCCTTACGGACTCCCCACC	GGAGATAGCTGCTCTACTTTGCC	0.99
KCNA1	116	GAGAATGCGGACGAGGCTTC	CCGGAGATGTTGATTACTACGC	0.88
MAPT	64	CGCTGGGCATGTGACTCAA	TTCTTCTCGTCATTTCTGTCC	1.01
MICOS10-NBL1/NBL1	101	CTCCCTGTCATGTTGCTGG	ACAATCTGCGTGATGTTCTTGG	1.05
MME	131	GGTTGAAACGCAACGTCATTC	CTGCACTGCTACTATGTCTTCAG	0.91
NOS1	114	ACGGCAAACCTGCACAAAGC	CGTTCTCTGAATACGGGTTGTTG	0.87
RORC	137	GACCCACACCTCACAAATTGA	AGTAGGCCACATTACACTGCT	0.97
S100B	84	TGGCTGCGGAAGTTGAGATT	ATGGCTCCCAGCAGCTAAAG	0.87
S1PR1	112	ATGGTGTCCACTAGCATCCC	CGATGTTCAACTTGCTGTGTAG	1.12
SELENOP	122	GTCCGTCTTCCCTCAGACAACC	GGGCTTTGTAACAAGCAGAGC	1.01
SULF1	104	TGTGTTCCACCGTTCGGTC	CACATCCTGGTCGTCAGTGAG	0.85
THBS3	156	ATGGAGAAGCCGGAACCTTTGG	AGTGAGTAAAGCTGTCCGAATCT	1.13
TMOD1	147	TGAGCTAGATGAACTAGACCCTG	CGGTCCTTAAATTCCTTCGCTTG	0.86
TNC	94	GCATCCGTACCAAAACCATCA	AACCCGTAGGGATTAGTGTCG	0.88
BIII-TUBULIN	92	GCGCCTTTGGACACCTATTCA	GCCCTCCGTATAGTGCCCT	0.99
TBP	84	CACAGGAGCCAAGAGTGAAGAA	TTAGCTGGGAAGCCCAACTTC	0.97
RPLP	109	CTCGCTTGTCATCTACTCCGC	AGAAAGGTTGACGCTGACAC	0.96

<sup>a</sup> 5'→3' sequence, E – efficiency values