

Effects of Chlorpyrifos Exposure on Proliferation and Differentiation
in Human Neural Progenitor Cells

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Abstract

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An *in vitro* human neural progenitor cell (hNPCs) model was established to study adverse effects of the organophosphate pesticide chlorpyrifos (CP) during neuronal proliferation and differentiation. CP is of interest given its association with altered neurodevelopment in children. Commercially available hNPC ENStem-A™ cells (Millipore) were expanded in serum free proliferation expansion medium (PEM) and differentiated in HyClone neural differentiation medium (HDM). Cells were pre incubated with PEM overnight, then incubated for 72 hours in either PEM or HDM with CP (0-200 µg/mL). Dose-dependent decreases in cell viability were observed in cells cultured in PEM and HDM, with significantly greater effects of CP on cells in the HDM (78%±3.2%) than in the PEM (84%± 3.6%) at concentrations of CP 20 µg/mL. Cells grown under differentiating conditions were more sensitive to CP.

Long-term cultures of hNPCs had significantly increased expression of neuronal structure markers (β -tubulin III, MAP2) as well as in neuronal functional marker (α -synuclein) over time under differentiating culture conditions. Simultaneously, proliferation marker (PCNA) expression decreased over time in long term differentiating conditions. Under proliferation cell culture conditions, there were no significant changes in expression of these neuronal markers. hNPCs were treated with CP (0-20 μ g/mL) under both proliferation and differentiation cell culture conditions and the effects on these neuronal stage markers were observed at 72 hrs. CP had a dose-dependent effect on expression of β -tubulin III, MAP2, and PCNA though there were no differential effects between the responses to CP under proliferation and differentiation culture conditions.

These results suggest that, while proliferating and differentiating hNPCs have different sensitivity to CP, common pathways of response are affected within both conditions. Alterations of neuronal protein markers during proliferation and differentiation by CP treatment indicate cell stage-specific molecular and cellular response outcome pathways and provide mechanistic clues for understanding the potential effects of environmental agents on different processes during neurodevelopment.

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Chapter 1: Introduction

Organophosphate (OP) Insecticides

The organophosphate insecticides are designed to be nerve agents intended to kill insects by inhibiting acetylcholinesterase (Costa 2008). Acetylcholinesterase (AChE) is the enzyme that cleaves acetylcholine in the synaptic cleft and OPs cause irreversible inhibition of this enzyme resulting in buildup of the neurotransmitter acetylcholine and overstimulation of post synaptic neurons or muscle cells. The enzyme is important in normal control of nerve impulse transmission from nerve fibers to central nervous system (CNS), muscle cells, glandular cells, and autonomic ganglia. Organophosphates also inhibit butyrylcholinesterase (BChE), but the physiological function of this enzyme is unknown. Both AChE and BChE are essential to nerve function in insects, humans, and many other animals (Costa et al. 2008).

Examples of OPs include parathion, malathion, fenthion, chlorpyrifos, and diazinon. These insecticides are of concern to human health because they are primarily neurotoxicants which show neurologic symptoms both in the central and peripheral nervous system by irreversibly inhibiting AChE that is critical to nerve function in both insects and humans. Although OPs degrade rapidly in the environment, humans can be exposed to OPs, resulting in adverse health outcomes. Following acute exposures, accumulation of ACh causes muscle weakness, muscle contraction, fatigue, runny nose, tears, increased saliva or drooling, sensory and behavioral disturbances, depressed motor function, and respiratory depression. Severe OP poisoning can result in seizures and death. Interestingly, children often have accentuated responses after OP exposure, including seizures (22%-25%) and mental status changes like lethargy and coma (54%-96%) (Zwiener and Ginsburg 1988).

Chronic exposure occurs among the industrial workers with regular contact with OPs through formulation, mixing, and/or application of pesticides. The workers and their children are populations of risk who have long-term exposure to the OPs. Chronic exposures to OP have adverse effects on neurological functions and can result in developmental disorders. Neurobehavior and neuropsychiatric disorders include disrupted learning, memory, depression, weakness, nervousness, and fatigue (Gershon and Shaw 1961; Ray and Richards 2001; WHO and IPCS 2001).

Chlorpyrifos

Chlorpyrifos [*O,O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate] is an organophosphate insecticide applied widely around the world. CP was first registered by the Dow Chemical Company in 1965 to control insects on various crops and household pests. The U.S. Environmental Protection Agency (EPA) has restricted the use of CP in residential use due to its potential as a developmental neurotoxicant (USEPA 2002). Continued use of CP in agriculture, however, remains controversial due to the potential for children to be exposed in agricultural communities (Berkowitz et al. 2003; Berkowitz et al. 2004; Handal et al. 2008). In the United States, CP is allowable on a variety of pome fruits. Department of Agriculture statistics indicate that Washington State leads US fruit crop production including but not limited to apples, sweet cherries, pears, and raspberries (USDA 2007), and CP was one of the most heavily used OPs on these crops (USDA 2007). The U.S. EPA classification for CP is Class II, moderately toxic by oral and inhalation exposure, and Group E, evidence of non-carcinogenicity for humans. The Reference dose (RfD) for CP is 0.003 mg/kg/day.

Exposure in Adults and Children

OPs can enter the body through dermal and oral ingestion (Garfitt et al. 2002; van der Merwe et al. 2006). The majority of OPs are used in agricultural purposes, thus agricultural communities have higher exposure than the general population (Lee et al. 2007; Muniz et al. 2008; Atherton et al. 2009; Kisby et al. 2009). Deficits in neurobehavioral performance have been observed in adult agricultural workers (Rothlein et al. 2006).

Exposure of children to CP is of great concern, because children are more susceptible to CP contaminated food, water, and air compared to adults on a body-weight basis. Also, exposure to OPs at levels considered to be safe for adults can result in permanent loss of brain function to the developing brain of the fetus and infants (National Research Council (U.S.) and Committee on Pesticides in the Diets of Infants and Children 1993; Eskenazi et al. 1999). Therefore understanding both the potential for enhanced sensitivity as well as higher exposure potential is important.

Dietary intake has been a major source of OP exposure in the general population of children (Curl et al. 2003). Children spend more time closer to the ground than adults and have more hand-to-mouth contact, increasing opportunities for direct ingestion of pesticide residues in dirt and dust. Children's early developmental processes are easily disrupted and they have more time than adults to develop chronic disease (Landrigan et al. 1999). While all children can be potentially exposed to pesticides, farm worker children are exposed at higher levels (Curl et al. 2002; Thompson et al. 2003; Coronado et al. 2006). Two additional exposure pathways for these children are parental take-home from work locations and closeness to spray locations (Fenske et al. 2000; Jaga and Dharmani 2003).

Children's metabolism is different from adults and their vulnerability increase as they are less able to detoxify chemicals such as OPs. (Bearer 1995; Mortensen et al. 1996). The developmental onset of the detoxifying esterase Paraoxonase (PON1) is delayed and adult levels for these enzymes are achieved after two years postnatally (Furlong et al. 2006). The

authors mention that many of the newborns and some mothers may have higher sensitivity to adverse effects of OP exposures.

In addition to enhanced potential for exposure, children exposed to OPs have shown evidence for developmental toxicity and behavior deficits due to enhanced susceptibility to neuronal damage (Jurewicz and Hanke 2008). A study of adolescent applicators aged 9-18 years has shown a dose-response between years of pesticide exposure and behavior deficits (Abdel Rasoul et al. 2008).

Exposures during Early Development

Early development is a critical period of vulnerability. Chemical exposure during a key “window of susceptibility” can disrupt organ formation and cause lifelong functional impairments (Rodier 1994). If exposure occurs during pregnancy, CP and its metabolites are detected not only in maternal urine, but also passing through the placental barrier and in cord blood and meconium (Whyatt et al. 2004; Engel et al. 2007; Ostrea et al. 2009).

The fetal blood-brain barrier is not fully developed, thus the fetal brain may be more concentrated with compounds than the brain of adults (Rozman and Doull 2001). During the prenatal period, the human brain develops from ectodermal cells into a complex organ made of billions of precisely located and specialized cells. Neurons must go through precise pathways and communicate with other cells for optimum development of the brain (Rice and Barone 2000). Development of the nervous system contains critical periods of vulnerability. Each developmental stage has to take place within a tightly controlled time frame. If any aspect of brain development is altered, the consequences may be permanent because there is little potential for repairing damaged brain cells (Rice and Barone 2000). *In utero* exposures to CP in humans can cause developmental delays in learning and mental and motor development (Engel et al. 2011). If brain cells are injured by CP during the prenatal period, the consequences can

include developmental disabilities, increased risk of neurological degeneration. Ample animal data suggest that exposure to pesticides during pregnancy and early life may impair growth and neurodevelopment in offspring (Li et al. 2012).

Adverse Neurologic Effects of Chlorpyrifos Following *in utero* Exposures in Humans

The developing nervous system is more sensitive and vulnerable to toxic chemicals compared to that of the adult brain. Exposure levels that have few or no effects in adults may pose serious risk to the developing nervous system (Faustman et al. 2000). Even at relatively low levels, OPs like CP can be hazardous to the brain development of fetuses and children, because they have lower levels of the detoxifying enzymes that deactivate OPs than adults (Furlong, Holland et al. 2006). The dynamics of AchE inhibition are complex. They are a product of time and exposure and the rate of AchE turnover as well as rate of OP metabolism and deactivation. (Padilla et al. 2000). Of concern for public health are the recent findings that prenatal exposure to CPs is associated with shortened gestational days (Rauch et al. 2012), smaller head circumference (Berkowitz, Wetmur et al. 2004), reduced birth weight and length (Berkowitz, Wetmur et al. 2004; Eskenazi et al. 2004; Zhao et al. 2005; Rauh et al. 2006; Wickerham et al. 2012) and found association of developmental delay at 3 years of age (Berkowitz, Wetmur et al. 2004; Zhao, Gadagbui et al. 2005; Rauh, Garfinkel et al. 2006; Wickerham, Lozoff et al. 2012). CP has been associated with reduced head circumference and anomalies in primitive reflexes (Whyatt, Rauh et al. 2004).

A Polish human cohort study has found significant increases in cases of miscarriage followed by occupational exposures to CP (Pastor et al. 2001). In addition, a positive association between occupational exposure to pesticide during early pregnancy and the risk of still births has been described by Pastore et al. (Pastore et al. 1997). Epidemiologic studies have

found an association between organophosphate exposure and reproductive disorders including infertility, birth defects, miscarriage and perinatal mortality (Baldi et al. 1998; Sanborn et al. 2002; Recio et al. 2005).

OP exposure is associated with increased ADHD (Marks et al. 2010) and decreased IQ or poorer intellectual development (Bouchard et al. 2011). Children prenatally exposed to CP also have an increased risk of developing attention disorders years later (Rauh, Garfinkel et al. 2006). A human cohort study found that the CP level measured in both umbilical cord plasma and the mother's plasma at the time of birth is correlated with increased risk of adverse effects, including developmental delay and attention problems at 3 years of age (Rauh, Garfinkel et al. 2006). This research suggests that children with prenatal exposure are more likely to develop symptoms of attention disorder and ADHD. This effect appeared to be stronger for boys than for girls (Horton et al. 2012). Another recent study reports significant associations of prenatal CP exposure with potentially irreversible brain structure abnormality in developing children brain (Rauh, Garfinkel et al. 2006; Rauh et al. 2012). Understanding when, how and at what doses CP exposures affect normal development is critical for our understanding and ability to predict rather than report impacts.

Adverse Neurologic Effects of Chlorpyrifos in Animal Exposure Models

Animal models have been utilized to study CP and have provided significant insight into windows of susceptibility. Numerous studies have shown that young animals are more sensitive than adults to the acute toxicity of chlorpyrifos (Eaton et al. 2008). For example, there is an approximately 6-fold difference in maximum tolerated dosages between neonate postnatal day (PND) 7 rats and adult rats treated with CP (Pope et al. 1991). The LD₅₀ of chlorpyrifos in neonate postnatal day (PND) 7 rats was reported to be 9-fold higher than the adult rats, LD₁₀ (Zheng et al. 2000). Moser and Padilla also reported a 5-7 times higher sensitivity in young rats

compared to adult rats (Padilla, Buzzard et al. 2000). A number of studies report behavioral abnormalities including changes in locomotor skills and cognitive performance in adolescent rats and mice after CP exposure (Dam et al. 2000; Ricceri et al. 2003; Icenogle et al. 2004).

Prenatal exposure studies with pregnant rats treated with chlorpyrifos (7 mg/kg/day, p.o.) on gestational days (GD) 14–18 showed similar peaks in terms of time of inhibition of brain AChE for the dams and the fetuses (Lassiter et al. 1998). A toxicogenomic study of maternal and fetal mouse brains exposed to CP 2-15 mg/kg/day on GD 6 to 17 reveals dose-dependent alterations in transcriptional response (Moreira et al. 2010). Another study shows that pregnant mice dosed with CP concentrations of 15.9, and 21.2 mg/kg between Gestation Day 6-15 have decreased litter size and pup weights. In addition, pups prenatally exposed to CP (15.9 mg/kg and 21.2 mg/kg) were born weak and died a few days postpartum (Ambali et al. 2009).

Mechanisms of Chlorpyrifos Toxicity

The toxic effects of CP are related to the ability of its oxon metabolite to bind and inhibit AChE in target tissues (Eaton, Daroff et al. 2008). CP can inhibit neuropathy target esterase (NTE) enzymes which are essential for placental development, blood vessel development and protein synthesis in the central nervous system (Lotti and Moretto 2005). Although AChE inhibition is known as primary mechanism for the CP toxicity, several *in vitro* studies suggest alternative neurodevelopmental mechanisms that are altered at concentrations of CP below cholinesterase inhibition (Pope 1999; Eaton, Daroff et al. 2008). Comparisons of gene expression patterns after exposures to different OPs reveal difference in neurotoxicological effects even though they all have the ability to inhibit AChE (Slotkin et al. 2007; Slotkin and Seidler 2009). These evidence suggest that the toxicological effects are exerted by non-AChE mechanisms as well. Potential molecular targets include inhibition of neurite outgrowth,

oxidative stress, and DNA damage (Eaton, Daroff et al. 2008). Changes in epigenetic factors may also be an underlying mechanism of CP effects.

In vitro neuronal outgrowth in primary rat neurons is inhibited at concentrations that do not inhibit AChE (Howard et al. 2005). The observation was explained with morphogenic rather than the enzymatic activity of AChE.

Other proposed mechanisms for CP include the induction of oxidative stress. Many studies have shown that CP increases oxidative stress or lead to oxidative damage to various tissue or cells (Oncu et al. 2002; Verma and Srivastava 2003; Slotkin and Seidler 2009). Initiation of oxidative stress is commonly associated with formation of an active metabolite or further metabolism that generates reactive oxygen species and lipid peroxidation (Verma and Srivastava 2001). CP promotes glutathione (GSH) depletion exhibited higher levels of cytotoxicity and markers of oxidative stress in a cell culture model of mice neurons (Giordano et al. 2007).

CP has the potential to cause DNA damage via indirect mechanism (Abdel-Rahman et al. 2002). Reactive oxygen species are a possible mechanism for this DNA damage. A number of non-AChE studies reflect that damage to the developing brain occurs at exposures below AChE inhibition and/or threshold for acute signs of intoxication (Slotkin 2004; Slotkin et al. 2005). Therefore, using the cholinesterase biomarker may not be adequate to monitor safety (Slotkin 2004; Mauro and Zhang 2007). Better understanding of the effects of CP on proliferating and differentiating cell populations during critical windows of susceptibility will improve risk assessment for exposures during key periods of development. For the thesis project, an *in vitro* human neural progenitor cell culture model is established and utilized to further evaluate the effects of CP on neuronal proliferation and early differentiation of hNPCs.

The Human Neural Progenitor Cell (hNPC) Culture Model

Human brain development must take place with precise timing in specific regions of brain. Development of the nervous system contains critical periods of vulnerability. If any aspect of brain development is altered, it results in lifelong irreversible damages in neuronal development (Rice and Barone 2000). It is important to evaluate for the environmental toxicant effects on these sensitive neurodevelopmental endpoints. Responses to environmental toxicants including chlorpyrifos vary with stages of development and with proliferation or differentiation status of the cells.

In vitro differentiation of neural progenitor cell (NPC) cultures provides opportunities to study the early stages of neuronal differentiation processes and is becoming a valuable tool in neurodevelopmental toxicology. (Shin et al. 2006; Radio and Mundy 2008). In our current model of neuronal differentiation, commercially available ENStem-A™ human neural progenitor cell (hNPC) was used to study the early stage of neuronal differentiation. Also, the differential effects of CP on the neuronal progenitor cells during proliferation and early stage of differentiation was evaluated. NIH-approved H9 (WA09) human embryonic stem cell (hESC) line is derived from the inner cell mass of discarded *in vitro* fertilization (IVF) embryo at the pre-implantation blastocyte stage with normal XX karyotype by Thomson et al (Thomson et al. 1998; Mitalipova et al. 2003). This H9 cell line was differentiated to ectoderm then to neural progenitor cells by Stice et al and made commercially available under the name of ENStem-A™ Human Neural Progenitor Cells (Millipore, Billerica, MA)(Shin et al. 2006; Dhara et al. 2008).

ENStem-A™ proliferates as adherent cell monolayers. This inherently proliferative feeder-free of neural progenitor cells is more than 90% positive for the neural progenitor marker Nestin, and less than 5% positive for the pluripotency marker Oct-4 (Thomson, Itskovitz-Eldor et al. 1998; Shin, Mitalipova et al. 2006). Withdrawal of fibroblast growth factor (bFGF2) and addition of neurotrophic factors leads these hNPC cultures to undergo a dramatic morphological

and functional transition to become neurons, oligodendrocytes and astrocytes (Young et al. 2011). hNPC are powerful research tools for the design and discovery of new approaches to neurodegenerative disease and developmental neurotoxicology. This line has already been employed to develop high throughput toxicological screening methods (Breier et al. 2008; Radio and Mundy 2008). In this thesis, the human ENStem-A™ hNPC culture is optimized and applied to further explore the developmental neurotoxicity and the mechanisms of chlorpyrifos (CP) during two stages of neural development, proliferation and differentiation. Changes in histone H3 modification sites were measured to evaluate whether alteration of proliferating or differentiating hNPC have association with epigenetic changes.

Hypothesis and Specific Aims

The developing brain is particularly vulnerable to environmental factors during early stages of cellular fate and specification. The *In vitro* human neural progenitor cell model is a promising tool for exploring the unique sensitivity of brain cells in these early stages of neuronal differentiation. The objectives of this work is to optimize an *in vitro* human neural progenitor cell (hNPC) model of early neurodevelopment and to use this model to study the effects of chlorpyrifos on proliferating and differentiating neural progenitor cells.

The hypotheses of this work are:

1. There is different sensitivity of proliferating and early differentiating hNPCs with chlorpyrifos treatment
2. Differentiating neuronal cells are more sensitive to chlorpyrifos exposure

The specific aims of this work are:

1. To develop a hNPC culture model to recapitulate normal developmental process of proliferation and differentiation *in vitro*
2. Exposing proliferating and differentiating hNPCs with CP

Chapter 2: Materials and Methods to Characterize hNPC Proliferation and Differentiation: Establishing the Model and Assessing the Effects of Chlorpyrifos Exposure during Proliferation and Differentiation of hNPCs

Introduction

To investigate the hypothesis, an *in vitro* human neural progenitor model was used. As proposed by ENStem-A™ *in vitro* Human Neural Progenitor Cells (hNPC) have strong potential to provide models for very early stages of nervous system development (e.g. neural tube and post neural tube early stages), to provide models for drug screening, toxicity, and to provide cell-based mechanisms for environmental toxicants (Martinez et al. 2012). Therefore, many investigators are determining if differentiation of neural progenitor cell (hNPC) cultures is a valuable tool for neurodevelopmental toxicity, providing a replicable yet dynamic model of neurodevelopmental processes (Shin et al 2006; Radio et al 2008). ENStem-A™ Human Neural Progenitor Cells obtained from Millipore Corporation (Billerica, MA). ENStem-A™ are derived from H9 (WA09) human embryonic stem cell line (Shin et al 2006). The reviews of human stem cell lines have identified and contrasted these cells versus other available cell lines. It has been reported that the ENStem-A™ (H9) cell line is derived from a normal female karyotype, is not genetically altered and has a normal complement of chromosomes following ten passages (Shin, Mitalipova et al. 2006; Ware et al. 2006). In our lab, we expanded cells up to passage 8 before performing each experiment. At this stage, ENStem-A™ hESC are greater than 90% positive for Nestin, a neural stem cell marker, and less than 5% remained positive for Oct-4, a hESC-marker. Upon withdrawal of fibroblast growth factor (bFGF2) from medium, hNPC has capacity to differentiate into a various neuronal subtypes or astrocyte lineages (Shin, Mitalipova et al.

2006).

Part 1 of the study is to characterize the neuronal differentiation and proliferation of hNPC during long-term culture. Our current study includes the observation of morphological changes and examining the changes of neuronal differentiation marker expressions over the course of 21 days of differentiation.

In Part 2, the thesis then focuses on the effects of chlorpyrifos exposure during the early stage of neural differentiation. The hypothesis of this study is that there is different sensitivity of neuronal cells during treatment with CP on proliferation and differentiation of hNPC and differentiating hNPC are more sensitive to CP exposure. Early CP exposure is expected to affect neuronal cell proliferation and differentiation by altering the critical signaling pathways and epigenetic factors. To test these hypotheses, the human neural progenitor cell cultures were set up on the laboratory and stable, replicable cell conditions were established. Dose and time-dependent effects of CP treatment were followed by observations on morphological changes, cell viability using Alamar Blue assay and Live and Dead Cell Staining at 24 hrs and 72 hrs after treatment. The protein expression levels of cellular signaling responses of neural differentiation markers are examined at 72hr.

Arsenic is well characterized as a potent developmental toxicant and a teratogen with *in vivo* and *in vitro* models, primarily inducing anterior neural tube defects (NTDs) (Willhite and Ferm 1984; Chaineau et al. 1990; Mirkes and Cornel 1992; Golub et al. 1998). In this study, Arsenic (As) is used as positive control for CP treatment.

Part I. Characterization of Non-treated Proliferation or Differentiation hNPCs over Long-Term Culture

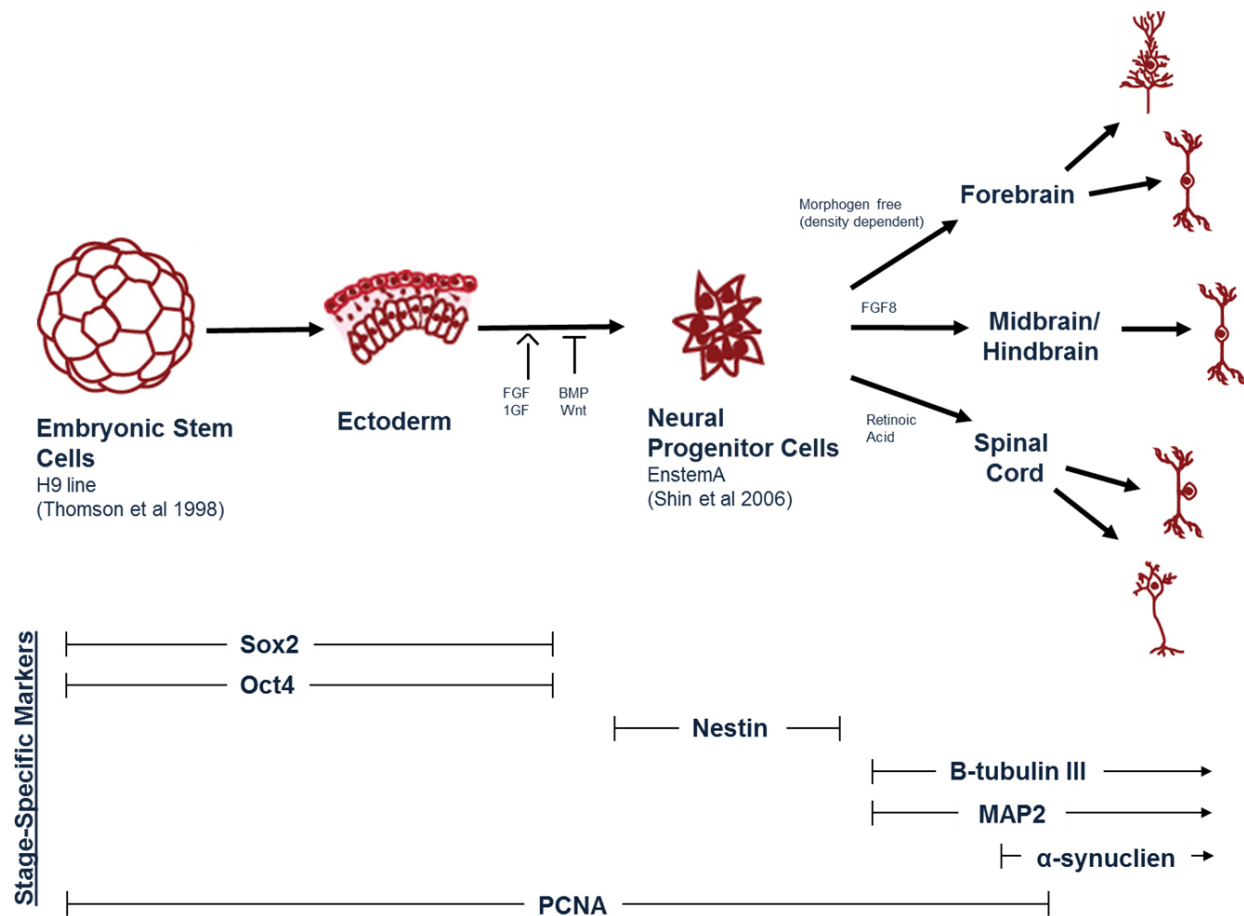


Figure 1 Neuronal differentiation and stage-specific markers

H9 human stem cell line is derived from human blastocysts. Among three germ layers, ectoderm gives rise to neural tissues. Neural progenitor cells are mediated by specific signal gradients such as fibroblast growth factor (FGF), insulin-like growth factor (IGF), bone morphogenetic protein (BMP) and Wnt molecule. NPCs are available commercially under product name ENStem-A (Millipore) and this provides a nice starting point for neuronal differentiation. Depending on the signals, the NPCs can differentiate to multiple regions of brain, and from there specific signals will lead more highly specified neuronal cell types such as glial, astrocyte, and oligodendrocytes.

Marker	Biological Function	Cells Detected
*Sox-2	Transcription factor essential for maintaining self-renewal	Embryonic Stem Cells
Nestin	Intermediate filament protein involved in radial axon growth	Neural Progenitor Cells
β -tubulin III	Component of microtubules found in cytoskeleton and axons	Structural Neuron Specific Marker
*MAP2	Microtubule associated protein located primary in dendrites	Structural Neuron Specific Marker
α -synuclein	Presynaptic marker	Functional synapsis Mature Neuronal Marker
*PCNA	Proliferating Cell Nuclear Antigen	Proliferating Cells

Figure 2 Markers of neural progenitor, differentiation, and functional maturation

Stage-specific markers used to track process through neuronal differentiation and functional maturation.

* Sox2 : Sex determining region Y box-2 * MAP2 : Microtubule-Associated Protein 2

* PCNA : Proliferating Cell Nuclear Antigen

hNPC Culture - Expansion

ENStem-A™ Human Neural Progenitor Cells (Millipore) were expanded as monolayers on the tissue culture treated polystyrene plates double coated with poly-L-ornithine (20 μ g/mL) and laminin (5 μ g/mL). These procedures followed the methods suggested by Stice (Shin, Mitalipova et al. 2006; Dhara and Stice 2008). Media were changed every other day with ENStem-A™ neural expansion media (Millipore) which is serum-free and feeder-free proliferation expansion medium (PEM), consisting of Neurobasal medium with B27 supplementation containing 2mM l-glutamine and 50 μ g/mL penicillin/streptomycin (Invitrogen), and 10ng/mL leukemia inhibitory factor (LIF, Chemicon) and fibroblast growth factor-2 (bFGF2,

R&D Systems) (Shin, Mitalipova et al. 2006). The bFGF2 and LIF in PEM help cells to adhere on the dishes. To passage the cells, cells were split in two flasks when they reached 90-100% confluence. Generally, it took about 3-4 days for the cells to reach full confluence after passaging.

For splitting, the cells were enzymatically detached from the flasks with Accutase (Millipore). The cells were then collected in a centrifuge tube and centrifuged at 200 X g (800 rpm) for 5 minutes. After centrifuging, the cells were resuspended in fresh PEM and replated in two new flasks. Cells were passaged in a humidified 37°C, 5% CO₂ incubator until reaching passage 8. Detailed spectral analyses indicate ENStem-A™ cells retained a normal diploid karyotype in culture after ten passages.

hNPC Culture - Experiment

Once passage 8 cells reached full confluence, cells were dissociated with Accutase (Millipore), and plated at a density of 200,000 cells/mL in 35mm dishes (400,000 cells per dish) or 96 black-bottom well microplates (20,000 cells per well) in proliferation expansion medium (PEM, Millipore) with presence of bFGF2 and LIF to allow cells to adhere in monolayer. After 24 hrs of plating the cells, media was refreshed to either proliferation expansion medium (PEM, Millipore) or HyClone differentiation medium (HDM, HyClone). HyClone basal Neural Differentiation Medium with addition of AdvanceSTEM Growth Supplement (HyClone) was used to induce differentiation. Basic composition of difference of HDM is removal of FGF2 and EGF. However, the exact medium composition is unknown because of commercial proprietary. For part 1 of the study, the cells were maintained in proliferation media for 3 days or in differentiation media for 21 days. For differentiating hNPCs, cells were replaced with fresh HDM on Day 3, then every other day until day 21. Morphology of the cells was observed and the protein was harvested to characterize the cellular signaling responses of neuronal development. By day 3, proliferating cells reach full confluence and cells start to detach from plates.

Differentiating cells, however, start to show extensive neurite outgrowth and start migrating to form neurosphere.

For part 2 of the study, the proliferating and differentiating hNPCs were treated with chlorpyrifos to study the adverse effects on these different cell types. Early differentiation is the stage of interest, thus we chose to observe effects at 72 hrs when the characteristics of proliferating and differentiating cells are noticeably different morphologically and start to show changes in neuronal marker expressions.

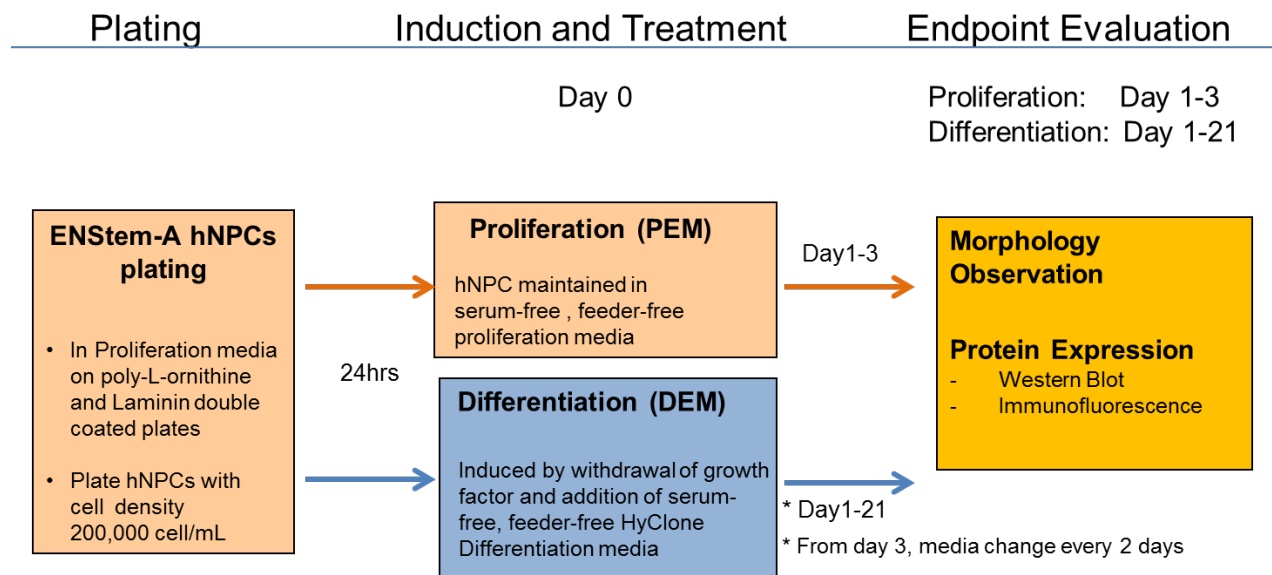


Figure 3 Methods of long-term culture of *in vitro* hNPCs proliferation and differentiation

ENStem-A™ hNPCs are plated in Proliferation Expansion Media (PEM) with density of 200,000 cells/mL and after 24hrs, the media was replaced with either PEM to continue proliferation or HyClone Differentiation Media (HDM) to induce differentiation. Endpoints of hNPCs in PEM were observed for 3 days and HDM for 21 days.

Morphology

All cell culture was monitored with a Nikon inverted microscope equipped with phase-contrast optics (Nikon, Tokyo, Japan) to assess the general morphology. Morphological images were captured and digitized with a Coolsnap Camera (Roper Scientific, Inc. Duluth, GA).

Immunofluorescence

Expression of stage-specific protein markers was observed in both treated and non-treated hNPC using immunofluorescence staining. The cells were plated with 200,000 cells/mL density in 35x10 mm dishes (total 400,000 cells per dish) in PEM and incubated for 24 hrs to let the cells adhere on the dish. Then the media was refreshed either in proliferation media (PEM) or differentiation media (HDM). For Part 1 of the study, Nestin (Proteintech) and β -tubulin III (Millipore) were used to examine the expression of neural progenitor stage and neuronal specific stage. Proliferating cells were fixed at 24 hrs (day 1) and 72 hrs (day 3), after establishing proliferation conditions and differentiating cells were fixed at 24 hrs (day 1), 72 hrs (day 3), day 5, day 10, day 14 and day 21 after establishing differentiation conditions. For Part 2, β -tubulin III (Millipore) was used to examine the changes of neuronal specific markers with treatment of CP.

To observe the phenotype expression changes over time using immunofluorescence, the media was removed, then washed gently with 1 mL of 1x PBS. After removing 1x PBS, 4% formaldehyde was added and incubated 30 minutes at room temperature to fix the cells. The fixative was aspirated followed by a rinse with 1 mL of 1x PBS. The dishes were preserved at 4°C. For immunostaining, the cells were washed twice with 1X PBS, blocked for an hour in blocking buffer (10% 10x PBS, 5% Normal serum of secondary antibody (e.g. Normal goat serum), 85% of distilled water, 0.3% of Triton X-100). After blocking, 400 μ L of primary antibodies, β -tubulin III (1:100, Millipore) and Nestin (1:100, Proteintech), were added to each dishes and incubated overnight at 4°C on the rocker. The primary antibodies were washed three

times with 1X PBS for 5 minutes each. Then 200 μ L of Image-iT enhancer were applied and incubate for 30 minutes on the rocker. Plates were incubated in 500 μ L of antibody dilution buffer containing secondary antibodies tagged with Alexa Fluor® fluorochromes (1:1000 dilution) for 1-2 hours at room temperature in dark condition. Diluted Hoechst 33342 dye (Hoechst 33342 (1mg/mL, [final]=2.5 μ g/mL) was added into each dish. After rinsing twice, images were captured using the Nikon Labophot 2 with fluorescence microscopes (Nikon, Tokyo, Japan) equipped with Nuance Software version 3.1 (Caliper Life Sciences, Hopkinton, MA). The magnification used was 40x. MetaMorph software version 7.7 (Molecular Devices LLC, Sunnyvale, CA) is used to process the digitalized images.

Western Blot Analysis for Neuronal Markers

The cells were plated with 200,000 cell/mL density in 35x10 mm dishes (total 400,000 cells per dish) in PEM and incubated for 24 hrs to let the cells adhere on the dish. Then the media was refreshed either in proliferation media (PEM) or differentiation media (HDM). Three sets of proteins of proliferating cells were harvested at day 1 (24 hrs), day 2 (48 hrs), day 3 (72 hrs) after establishing proliferation conditions. Three sets of protein were harvested for at least three replicating experiments at day 1 (24 hrs), day 2 (48 hrs), day 3 (72 hrs), day 5, day 10, day 14 and day 21 after establishing differentiation conditions. Protein is harvested using 40 μ L of 1x cell lysis buffer containing 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na₂EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM β -glycerophosphate, 1mM Na₃Vo₄, 1 μ g/mL leupeptin (Cell Signaling). Harvested samples were immediately frozen in dry ice to prevent denaturing. After three freeze-and-thaw cycle, each sample were sonicated for 10-15 seconds then spun down for 5 minutes at 13.3 rpm to remove the cell pellet. After quantifying protein using Bradford protein assay, the western samples were prepared to load same amount of proteins (~11.25 μ g/15 μ L) for Western blots. Western blots were done to characterize the stage-specific protein expression changes. Primary antibodies used were Nestin (1:1000;

Proteintech), β -tubulin III (1:1000; Millipore), Microtubule-associated protein-2 (MAP-2) (1:1000; Proteintech), and α -synuclein (1:1000; Epitomics). For the proliferation marker, Proliferating Cell Nuclear Antigen (PCNA) (1:5000; Millipore) was used. Apoptosis marker, Caspase 3 (Cell Signaling) and stress marker, pMAPK p42/p44 (Cell Signaling) were also evaluated. To measure the internal standard for protein loading, β -actin (1:10,000; Sigma) was used.

Comparisons of hNPCs, Mouse Micromass Culture, and Other *In vivo* Systems

Human neural progenitor cells were evaluated as a model for human brain development. In order to study the relevance of our *in vitro* hNPC model of early neuronal differentiation to particular regions and stages of *in vivo* brain development, we compared the characteristic of proliferation and differentiation of the hNPC model with our previous *in vitro* micromass mouse embryonic neural progenitor cell model and data from other *in vitro* systems. Our lab has previously isolated embryonic neural progenitor cells from midbrains of E11 mouse embryos to study the developmental neurotoxicity. *In vivo* neuronal expression data is available at the Allen Brain Institute Developing Mouse Brain Atlas. We accessed their data to compare the changes of expression in neuronal markers in our *in vitro* hNPC culture system. The changes we observed in hNPC over the time course capture processes that are similar to those observed *in vivo* brain development. This provides an exciting system to provide a dynamic model to represent neuronal development processes.

Part II. Effects of CP Exposure on Proliferation or Differentiation hNPCs

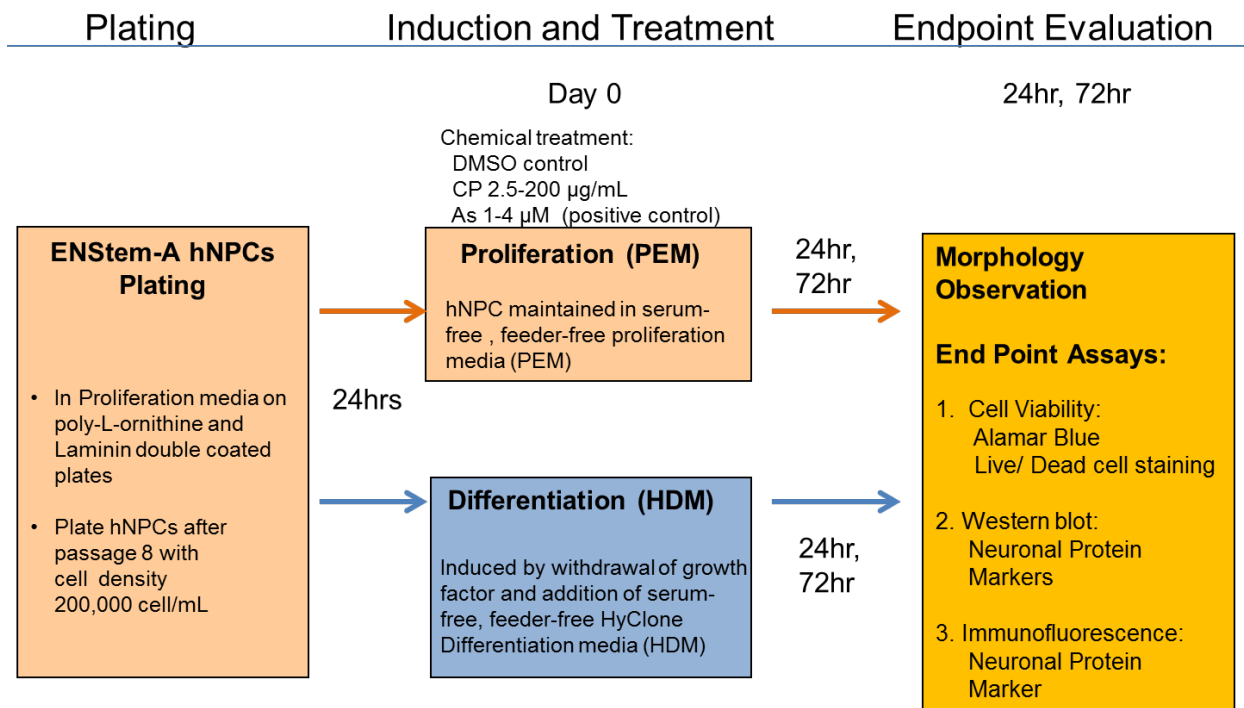


Figure 4 Experimental design of CP treatment.

ENStem-A™ hNPC are plated with density of 200,000 cells/mL and after 24hrs, treated with CP or As (positive control) either in PEM or HDM. Treatments are followed by end point assays as described in the boxes.

Experimental Design

hNPC, at passage 8, were harvested once reaching confluence of 90-100% and the number of cells was counted using a hemocytometer. Cells were seeded with 200,000 cells/mL in poly-L-ornithine (20 µg/mL) and laminin (5 µg/mL) double coated tissue culture treated polystyrene 35x10 mm dishes (2mL or 400,000 cells per dish) or 96-well black bottom microplates (100µL or 20,000 cells per well) (BD Falcon, New Jersey). After 24 hrs incubation in humidifying 37°C, 5% CO₂ incubator, the hNPCs were treated with Chlorpyrifos in Proliferation media (PEM) (Millipore) or HyClone Differentiation Media (HDM) (Thermo Scientific). Arsenic is used as positive treatment control.

Chemical Treatment

Chlorpyrifos (99.5% purity, ChemService, West Chester, PA) was dissolved in dimethyl sulfoxide (DMSO, Sigma St. Louise, MO) for rapid and complete absorption (Whitney et al. 1995). Stock solution of 100mg/mL and 10mg/mL were prepared so that Dimethyl sulfoxide (DMSP, Sigma, St. Louise, MO) did not exceed a final volume concentration of 0.1%. The stock solutions were made freshly for each experiment and treated within 30 minutes. Control cultures (CP0 $\mu\text{g/mL}$) were also applied with same volume of DMSO as added in the CP or As treatment. Calculated volume of the CP stock solution (100mg/mL or 10mg/mL) plus additional DMSO was added to the PEM or HDM stocks to make final CP concentration of 0-200 $\mu\text{g/mL}$ (or 0-570 μM) with same volume of DMSO loaded. For the dose-response cell viability study using Alamar Blue and Live and Dead Cell Staining, wide range of CP concentration, 0-200 $\mu\text{g/mL}$ (or 0-570 μM), were evaluated. CP concentrations of 0, 5, 10, 20 $\mu\text{g/mL}$ (or 0, 14, 28, 57 μM) were used for cell viability using 3 color assay and protein expression. The final concentration was chosen for the direct comparison to our lab's rodent *in vivo* study (CP concentration used 0-15 mg/kg) (Moreira et al. 2010).

Arsenic was used as a positive treatment control. Arsenic is well-known teratogen inducing embryo toxicity and neural tube defects (Robinson et al. 2011). Sodium arsenite (As^{3+}) was dissolved in DNase/RNase-Free Distilled water (Invitrogen) to make 100 μM concentration. The stock was serial diluted to make final concentration of 1, 2, or 4 μM . DMSO was also applied to all arsenic treatment media with the same volume of DMSO added in control and CP treatment groups. These concentrations were chosen based on our previous *in vitro* study which tested 0-20 μM on p53 +/+ and -/- cells (Yu et al. 2008).

Morphology

Morphological changes were observed as described in Part I. Morphology.

Cell Viability using Live and Dead Cell Staining (3 color assay)

To determine the viability of the cultured hNPCs, a three-color fluorescence assay was utilized to determine the number of live and dead cells. Non-fluorescent Calcein AM (Invitrogen) dye permeates into the live cells and are hydrolyzed to green, fluorescent Calcein by the intracellular esterase. Propidium Iodide (PI, Invitrogen) is not permeable to live cells, and selectively stains nuclei of damaged or dead cells with increased membrane permeability. PI has red fluorescence. Hoechst 33342 is used to stain the total nucleus of living and dead cells with blue fluorescence. The nuclei of live cells are evenly stained, whereas damaged or dead cells have intense and irregular staining.

The live and dead cell staining (3 color assay) procedure is followed as described in our previously developed system (Yu et al. 2005). Calcein AM (100 μ M, [final]=1 μ M), Hoechst 33342 (1 mg/mL, [final]=2.5 μ g/mL), and Propidium Iodide (1 μ g/mL, [final]=10 μ g/mL) were added directly into the hNPC cultures in 35mm dishes and incubated at 37 $^{\circ}$ C for 5 minutes. The fluorescence of the dyes in hNPC cultures were viewed using appropriate bandpass filters for Calcein AM (DM510, emission wavelength 560nm), Hoechst 33342 (UV, emission wavelength 460nm), and PI (DM580, emission wavelength 650nm) with the Nikon Labophot-2 with fluorescence microscopes (Nikon. Tokyo, Japan). Each of the images were captured and digitalized with a Spot Camera (Diagnostic Instruments, Sterling Heights, MI) equipped with Nuance Software Caliper Life Sciences. Hopkinton, MA. The magnification used was 20x. MetaMorph software (Molecular Devices LLC. Sunnyvale, CA) was used to process the digitalized images.

The live and dead cell staining (3 color assay) was conducted in one or two dishes from control, chlorpyrifos, and arsenic treatment group. In each dish, images were captured at three random views. The morphological changes were analyzed qualitatively. The number of Hoechst 33342 stained nucleus were counted automatically with the Cell Counting option in the Image J software (National Institute of Health, Maryland, USA). The live cells are Calcein AM positive

and dead cells are PI positive. Aside from live and dead cells, cells are categorized to be in process of apoptosis or injured when a cell has compromised membrane therefore expressing mixture of Calcein AM and PI, cell have membrane vesicle and blebs of membrane, or stained with Calcein AM but has bright, small condensed cells. Example of characterization of each cell types is explained in Figure 5.

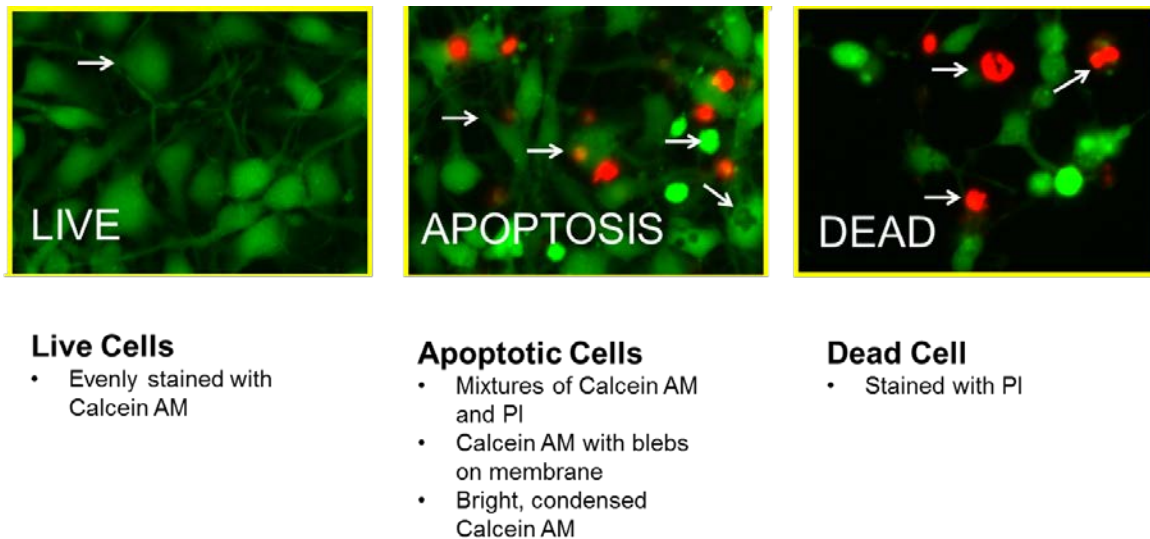


Figure 5 Characterization of live, apoptotic, and dead cells

Examples of characterization of live, apoptotic, and dead cells. The arrows specifically points to the descriptions under each images. This general rule was used to manually count cells of each type.

Cell Viability Assay (Alamar Blue)

After 24 hrs or 72 hrs of treatment, dose-dependent cell viability was measured using the Alamar Blue Assay (Invitrogen). Alamar Blue measures mitochondrial reductase activity of live cells by reducing non-fluorescent, blue resazurin dye to fluorescent, red resorufin (O'Brien et al. 2000).

Black 96 well microplates double coated with poly-L-ornithine (20µg/mL) and laminin (5µg/mL) were used for the Alamar Blue assay. After plating ENStem-A™ cells with 20,000 cells/well and incubating for 24 hrs in proliferation media (PEM), the media was replaced with

chlorpyrifos either in PEM or HDM. For dose-response studies, varying concentrations of CP from 0-200µg/mL and Arsenic 1-4 µM were incubated for 24 hrs and 72hr. 10% Alamar Blue (v/v) was added directly in each well 3 hours prior to 24 hrs and 72 hrs and incubated at 37°C in a 5% CO₂ incubator. Therefore, Alamar Blue assay measures for the live cells that are attached and floating in each well. After incubation, the 96 well plates were read on the fluorescence machine at 570nm with reference wavelength 630nm.

Immunofluorescence

hNPCs were exposed to CP (0-20 µg/mL) under proliferating and differentiating cell culture conditions. Expression of differentiating neuronal marker (β-tubulin III) and nucleus (Hoechst 33342) was observed. Detailed procedures are described in Part I.

Immunofluorescence.

Western Blot Analysis for Neuronal Markers

To characterize protein expression changes, western blots were done with 1) proliferation marker; Proliferating Cell Nuclear Antigen (PCNA), 2) neuronal progenitor cell marker, Nestin, 3) differentiating neuronal markers; β-tubulin III, Microtubule Associated Protein 2 (MAP2), α-synuclein, 4) apoptosis marker; Caspase 3, 5) stress marker; pMAPK p42/p44, and 6) β-Actin was used as loading control.

Proliferating and differentiating hNPCs were collected 72 hrs after treatment and were rapidly lysed in protein lysis buffer containing 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na₂EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na₃Vo₄, 1µg/mL leupeptin (Cell Signaling). Cells were gently washed once with 1X PBS and harvested by scraping in 40uL of cell lysis buffer. The cell lysate was repeatedly frozen and thawed three times and the insoluble materials were removed by centrifugation (13,000 rpm for 5 minutes at 4°C). The protein concentration was measured by using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Once the western samples were prepared, they were heated in

boiling water for 5 minutes then 10 µg of protein per lane (15µL) was loaded on 15 well x 1mm thick SDS-PAGE (Invitrogen). Gels were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore) using a vertical transfer apparatus. Membranes were rinsed briefly in Tris-buffered saline (TBS), pH 7.6. Non-specific binding of the blotted membrane was blocked with 5% non-fat-dried milk in TBS with 0.1% Tweens-20 (TTBS) for one hour, followed by incubation with primary antibody overnight at 4°C.

Following antibody incubation, the membrane was washed three times for 5 min with TTBS and incubated 2 hours with a secondary antibody. After hybridization with secondary antibodies conjugated to horseradish peroxidase, the immunocomplex was detected with the ECL detection reagent (Amersham Pharmacia Biotech) and exposed to X-ray films.

Primary antibodies used were Nestin (1:1000; Proteintech), β -tubulin III (1:1000; Millipore), Microtubule-associated protein-2 (MAP-2; Proteintech) (1:1000) and pMAPK p44/42 (1:1000; Cell Signaling). For the proliferation marker, Proliferating Cell Nuclear Antigen (PCNA) (1:5000; Millipore) is used. Cell apoptosis level was evaluated with Caspase 3 (1:1000; Cell Signaling). To measure the internal standard for protein loading, β -Actin (1:10,000; Sigma) was used.

Band intensity was quantified by densitometry using Image J software (National Institute of Health, Maryland, USA) and normalized to β -Actin. Arsenic was used as a positive control for our studies.

Statistical Analyses

Analysis of variance (ANOVA) was performed to assess the significance of data. For cell viability assays and neuronal protein markers, mixed effect model analysis was used to investigate significance differences between proliferation and differentiation cell culture conditions with CP or As exposures. Dose and cell culture conditions were fixed variables and the experiment preparation dates were used as a random variable effect. The interaction

between the fixed effects of dose and cell culture conditions was used to test whether there was a difference between cell culture conditions (i.e. under proliferating or differentiating cell culture conditions). One Way ANOVA was used to evaluate the cell responses across CP or As doses within each cell culture condition. Dunnett t-tests were conducted to determine significant difference between the individual treatments and control within each cell culture condition. A p-value of less than 0.05 ($p \leq 0.05$) was considered significant. In figures, asterisks are used to indicate different levels of statistical significance (“*” for significance at $p \leq 0.05$ and “***” for a statistical significance at $p \leq 0.001$, respectively). All statistical analyses were performed in SPSS version 18 (SPSS Inc. Chicago, IL).

The results of quantitative analysis of cell viability using Alamar Blue and Live and Dead Cell staining (3 Color Assay) were presented as average percent of controls (total cells) \pm SE (standard error of the mean). For Alamar Blue, four to eight control or treatments wells per experiment were used. For 3 Color Assay, one to two control or treatments dishes per experiment were used and the images were captured at three different locations per dish.

For Alamar Blue data analysis, a single blank was run each time the analysis was run. The blank media fluorescence measurement was subtracted from fluorescence measurements of cells from the same run. The percentage normalized by controls was calculated by dividing the adjusted fluorescence levels of tested cells to the adjusted level of control cells.

(*FL : fluorescence level)

$$\frac{*FL \text{ treated or control cells} - FL \text{ blank}}{FL \text{ control cells} - FL \text{ blank}} * 100$$

For the 3 Color Assay, total, live, dead and apoptotic cells were counted manually. Total cell counts for each treatment were normalized by 24hr control to assess dose response. In addition to effect of treatment on total cell counts, the proportion of live, dead, and apoptotic

cells were compared to understand the proportion of each cell types in the CP treated proliferation and differentiation hNPCs.

For neuronal and stage specific protein marker expressions, results for Western Blot band densitometric quantification is presented as average fold change to controls \pm SE. Each of the proteins analyzed in this paper was first normalized to corresponding β -Actin, then to corresponding controls. For Western blot, three control and treatments dishes per experiment were used.

In all three assays (Alamar Blue, 3 Color Assay and Western Blot) conducted for this paper, at least three separate experiments were performed for each treatment.

Chapter 3. Results: Effects of Chlorpyrifos Exposure on Proliferation and Differentiation of Human Neural Progenitor Cells

Summary

Part I of the study characterized neuronal differentiation and proliferation of the hNPCs derived from the NIH approved H9 embryonic stem cell line. We measured expression of neuronal differentiation markers using western blotting and immunofluorescence. We compared our *in vitro* human hNPC culture system to our previous *in vitro* mouse micromass data and the expression of neuronal markers in developing mouse midbrain presented by the Allen Brain Institute.

Five markers associated with neuronal differentiation were used to evaluate the expression of stage-specific neural proliferation, differentiation, and functional maturation of our hNPC cultures. These markers were PCNA (Millipore), Nestin (ProteinTech), β -tubulin III (Millipore), MAP2 (ProteinTech) and α -synuclein (Epitomics). In addition, apoptosis marker, Caspase 3 and stress marker, pMAPK p42/p44 were used. The total protein was measured using a structural protein, β -Actin (Sigma).

The results on the protein cell signaling expression of hNPCs showed that PCNA was expressed in both proliferating and differentiating conditions with a decreasing trend in later time points in differentiation culture (Day 14 and 21), suggesting that even in early differentiating conditions cells continue to proliferate.

β -tubulin III increased over time under differentiation conditions, with dramatic increases at Day 14 and 21. No significant changes of β -tubulin III was observed in proliferating conditions. MAP2 although less expressed than β -tubulin III, consistently increased in differentiation culture conditions, with minimal expression in proliferating cells. Expression of the

neural progenitor cell marker, Nestin, did not appear to change over time in Western blots.

In immunofluorescence, Nestin is only expressed at 24 hrs after differentiation and had no expression in later time points. β -tubulin III increased in immunofluorescence over time. We observed that cells migrate and form neurospheres and extended neurite outgrowth over time.

Part II of this research studied the effects of chlorpyrifos (CP) on proliferation and differentiation of human Neural Progenitor Cells (hNPCs) using Arsenic (As) as positive control. Our interest is in studying the effects of the CP and As on brain development during the early period of pregnancy. Neural differentiation is an important process throughout many stages of pregnancy and the timing varies by the brain regions. We were interested in early stages of neuronal differentiation. Therefore, we differentiated our hNPCs for 72 hrs where the cells start to express the neuronal differentiation markers.

We found different effects of CP during proliferation versus differentiation of NPCs. hNPCs were expanded in serum-free, feeder-free proliferation expansion medium (PEM) or differentiated in neural differentiation medium (HDM). Cells were incubated for 72 hours in either PEM or HDM with CP (0-200 $\mu\text{g}/\text{mL}$). Dose-dependent decreases in cell viability were observed in both cells cultured in proliferation PEM and differentiation HDM, with significantly greater effects of CP on cells in HDM ($78\% \pm 3.2\%$) than in the PEM ($84\% \pm 3.6\%$) at concentrations of CP 20 $\mu\text{g}/\text{mL}$. Cells grown under differentiating conditions were more sensitive to CP. We observed significant decreases in protein expression of PCNA and β -tubulin III in proliferation with increasing CP exposure. Our results demonstrate a differential effect of CP on neuronal cells during proliferation and differentiation. Differential alterations of neuronal protein markers during proliferation and differentiation by CP treatment may provide cell stage-specific molecular and cellular mechanistic clues for understanding the potential effects of environmental agents on the neurodevelopment.

Part I. Characterization of Non-treated hNPCs Proliferation or Differentiation over Long-term Culture

Results

Morphology of hNPCs Cultured in Proliferation and Differentiation

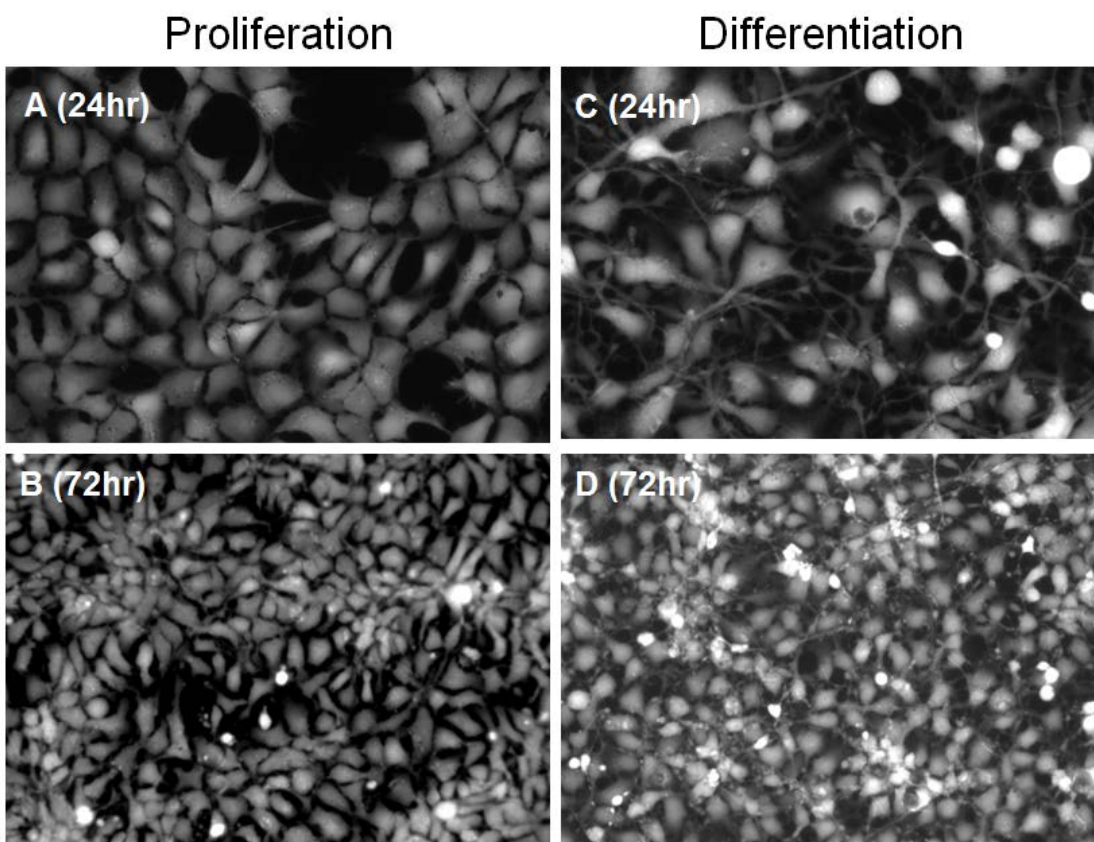


Figure 6 Morphology of hNPCs under proliferating or differentiating cell culture conditions

Morphology of hNPCs in proliferation media (PEM, left) or differentiation media (HDM, right) using high-magnification fluorescence-based photographs of live/dead cell assay at 24hrs (Top) and 72hrs (Bottom). Details are explained in the *Materials and Methods* section. Images in this figure represents the live cells by the Calcein AM uptake. Morphology is captured with 20x magnification. Note in the HDM condition, cells start differentiating and neurite outgrowth is observed at 24hr. Cells proliferate both in PEM and HDM condition. Images are representative of at least three independent experiments.

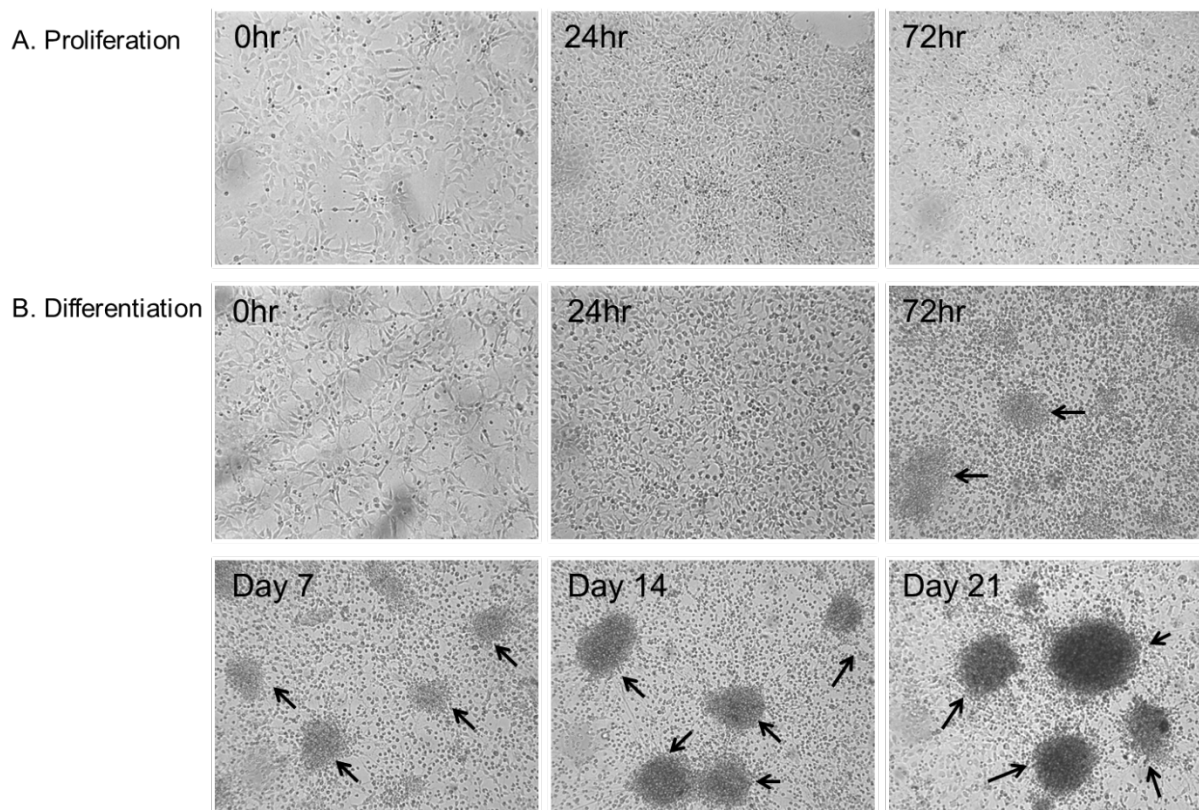


Figure 7 Phase-contrast morphology of non-treated hNPCs cultured under proliferating or differentiating cell culture conditions

Phase-contrast morphology of non-treated hNPCs cultured in proliferation media (PEM, A), or differentiation media (HDM, B) is shown with 10x magnification. hNPCs were grown and plated as described in *Materials and Methods* and seeded at 4.0×10^5 cells per dish in PEM. Zero hour indicates the time of initiating differentiation and refreshing proliferation media. Media was refreshed every other day after 72hrs. From left to right indicates the time of incubation. Under proliferation cell culture conditions, hNPC start to detach from plate once it reaches full confluence, therefore hNPC were allowed to proliferate for up to 72hr. Under differentiation cell culture conditions, continuous proliferation is observed and when cells reach full confluence, the cells migrate to form network and neurospheres (arrows). The cells are differentiated for up to 21 days. Images are representative of at least three independent experiments.

hNPCs were observed for 72 hrs of proliferation or 21 days of differentiation.

Proliferating cells reach full confluence at 72 hrs and start dissociating from the dishes. After 24 hrs of incubation in differentiation culture (HDM), we observe prominent neurite outgrowth in HDM differentiation conditions (Figure 6). Differentiation cells also reach full confluence at 72 hrs of incubation, but they migrate to form clusters of cells which later become ball-shaped

neurospheres. This cell shape and neurite growth is similar to what we have observed with our previous *in vitro* murine cortical neuronal cultures. After 72 hrs, this neurite outgrowth is more extensive in differentiating hNPCs. hNPCs reaches full confluence at 72 hrs under both proliferating and differentiating cell conditions, suggesting that proliferation continues under both culture conditions.

Immunofluorescence Expression of Nestin and β -tubulin III

Immunofluorescence expression of stage-specific neural markers in differentiating ENStem-A™ NPCs reveal a dramatic transition in differentiating hNPCs over time (Figure 8). Nestin, a neural stem cell marker is expressed at 24 hrs, but is not present at 72 hrs or at later time points. β -tubulin is a structural protein and β -tubulin III is a neural lineage specific marker. Increased β -tubulin III expression over time indicates progression of neuronal differentiation. From day 10 and beyond, there are clusters of β -tubulin III-positive differentiated cells with extensive neurite outgrowth.

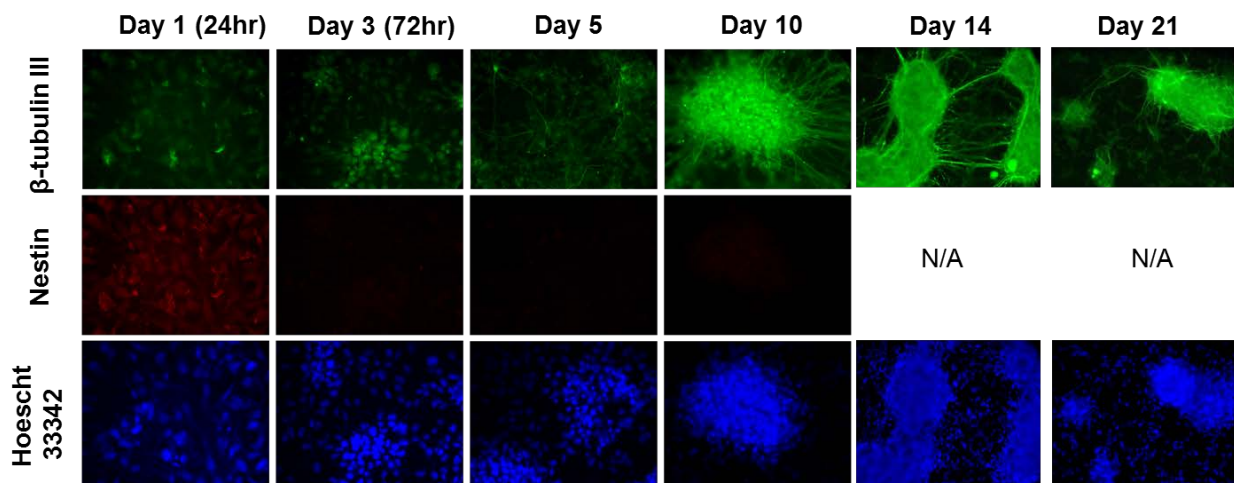


Figure 8 Immunofluorescence of differentiating hNPCs over time

Visualization of IF on differentiating hNPC over time (Magnification 40x). Differentiating ENStem-A™ cells were fixed, permeabilized and stained with fluorescent antibodies for neuronal-specific marker β -tubulin III and early neuronal stem cell marker Nestin. Nuclei were stained with Hoechst 33342. Changes in morphology and expression of neuronal markers are evident over time. Note that Nestin is expressed in the early time point (24 hrs), and beta tubulin III has increasing expression at the longer time of incubation.

Long-Term Protein Expressions of Different Neuronal Stages

Western blot analysis shows the expression changes of neuronal markers. As described in the Chapter 2. *Materials and Methods*. Five markers associated with neuronal differentiation were used to evaluate the status of our hNPCs, PCNA, Nestin, β -tubulin III, MAP2 and α -synuclein. While expression of these protein markers remains fairly static in proliferating conditions, expression of neuronal markers dramatically increases in differentiating cells over time in culture (Figure 9). Proliferating marker, PCNA is expressed in both proliferating and differentiating conditions with a decreasing trend in later time points in differentiation culture (Day 14 and 21) (differentiation $p=0.0244$). Neuronal marker, β -tubulin III is minimally changed in expression in proliferating conditions, but significantly increases in differentiating cultures at all times tested having about 7 folds increase at day 21 compared to day 1 (differentiation $p=0.026$). In addition, expression of α -synuclein, a marker of synapse formation, dramatically increases in differentiating cells over time in culture with about 12 folds increases by day 21 (differentiation $p=0.001$). Another neuronal marker, MAP2, although less expressed than β -tubulin III, consistently increases in differentiation culture conditions, with minimal expression in proliferating cells (differentiation $p\leq 0.001$). Expression of the neural stem cell marker, Nestin, do not appear to change over time under both proliferation and differentiation cell culture conditions.

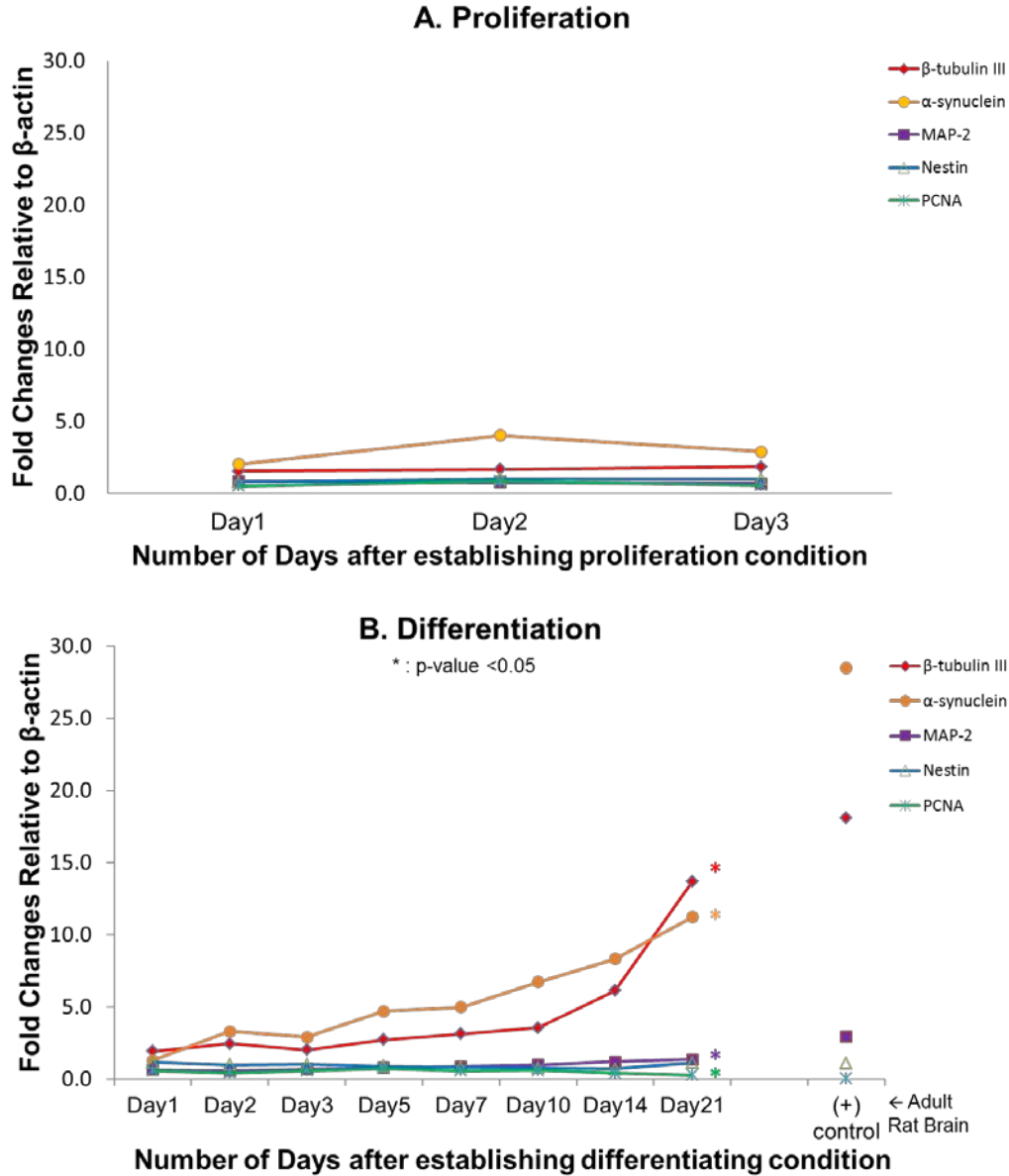


Figure 9 Characterization of protein expression markers in hNPCs over time and under proliferating and differentiating cell culture conditions

hNPC cells were cultured under proliferation and differentiation cell culture conditions to examine culture dynamics. Neuronal stage specific protein markers were evaluated on A. proliferation (day 1-3) and B. differentiation (day 1-21) of hNPCs using Western blots. Adult rat brain is used as positive control. β -actin was used as internal control and graphs represent the fold changes relative to day 0. While proliferation hNPCs has minimal changes of expression, differentiation hNPCs increase neuronal structural differentiation marker expression, β -tubulin III ($p=0.026$) and MAP2($p\leq 0.001$); increase functional maturation marker, α -synuclein ($p=0.001$); and decrease proliferating marker, PCNA ($p=0.0244$). No significant changes is observed in neural progenitor marker, Nestin. Statistical analysis was done using One Way ANOVA for each cell culture condition over time. Data is representative of at least three independent experiments.

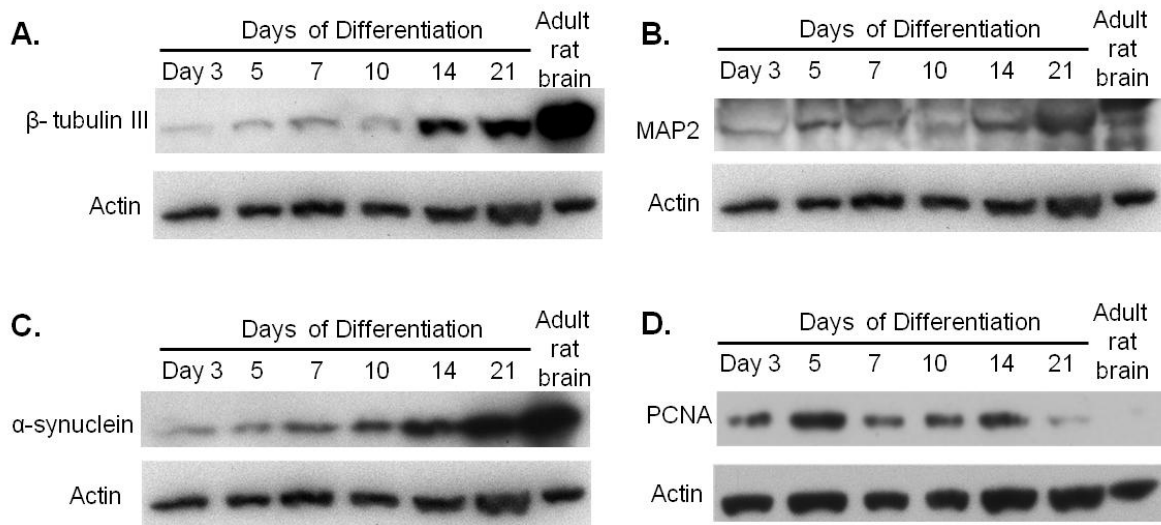


Figure 10 Illustration of cellular signaling responses in hNPCs under differentiating cell culture condition on Day 3-21 using Western Blots

Here, the Western Blot bands illustrate that neural lineage markers β -tubulin III and α -synuclein are increasing its expression dramatically at later time points (data results shown in Figure 9). MAP2 also increases over time. Proliferation marker, PCNA is expressed in earlier days then decrease at later time points. Data is representative of at least three independent experiments.

Comparisons of hNPCs, Mouse Micromass Culture, and Other *In vivo* Systems

Allen Brain Institute has dynamic changes in expression of markers of differentiation over time are clear in each model. Normal brain development process was plotted and compared to expression observed in two different *in vitro* culture systems used in Faustman lab. The pattern of β -tubulin III expression during E13.5-18.5 appears to be similar to that in hNPC cultures Day 2-Day 21 *in vitro*. Similarly, the decrease in Nestin expression seen early *in vivo* is evident in micromass cultures after a few days in culture. Differentiating hNPCs also had minimal expression of Nestin. However, each model displays a unique pattern of expression. Although the change of MAP2 expression is not consistent with the increases shown in hNPCs, both agree that it has lower expression compared to other neuronal specific markers.

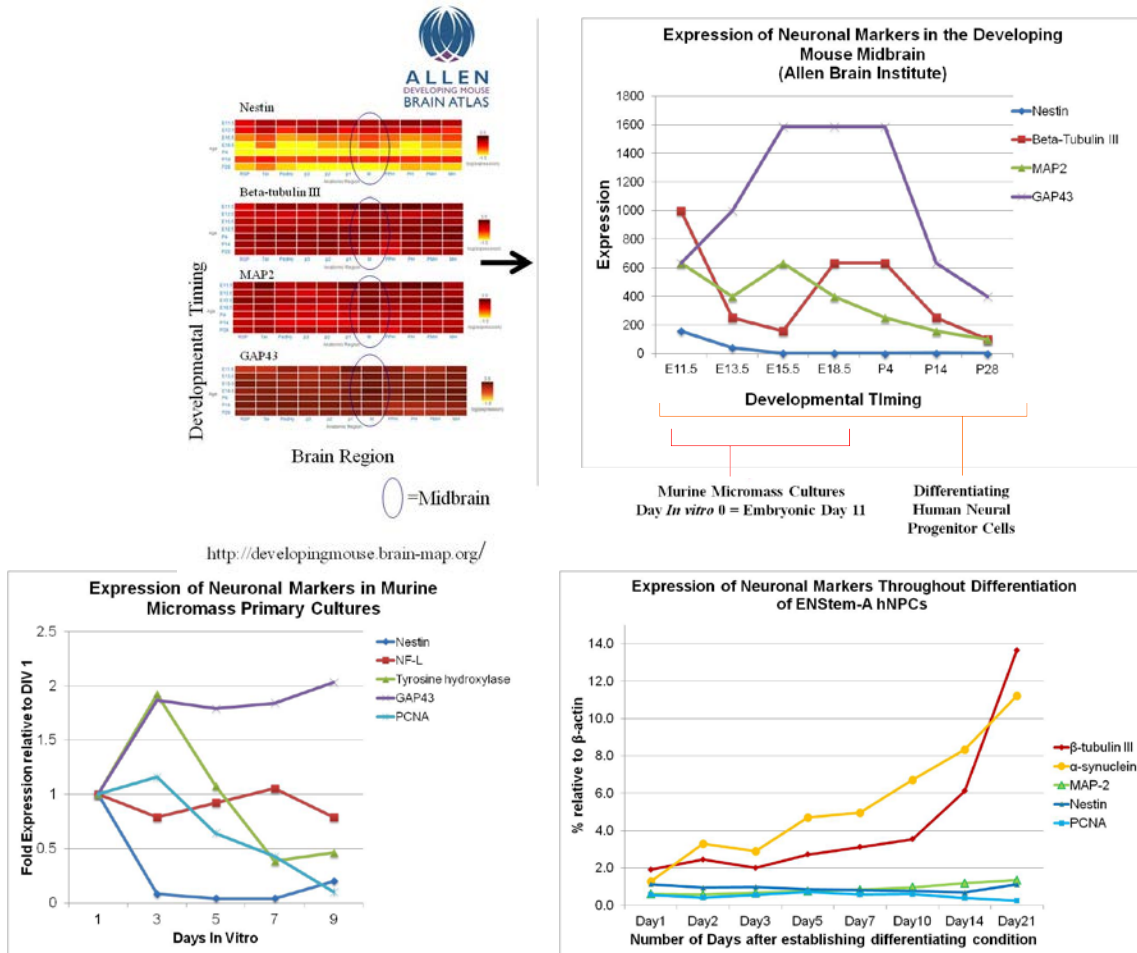


Figure 11 Comparison of expression across time in *in vivo* and *in vitro* systems

Data from the Allen Brain Institute on expression of neuronal markers in murine brain development was plotted and compared to expression observed in two different *in vitro* systems used in the Faustman lab. Dynamic changes in expression of markers of differentiation over time are clear in each model. Allen Brain Institute data source: <http://developingmouse.brain-map.org/>

Discussion and Future Direction

Human brain development is a precise and complex process. Disruption during critical periods of vulnerability of CNS development can result in lifelong neuropathologies.

Understanding responses to environmental risk factors and their mechanisms of action will

improve our capacity to identify the toxicological profile of many unknown developmental

neurotoxicants. *In vivo* studies are commonly used as the guideline for evaluation of neurotoxic

effects. However, *in vitro* neurotoxicity tests are an important alternative method to *in vivo* studies to evaluate large sets of chemicals. Thus far, studies have focused on a limited set of descriptive endpoints, including cell viability, apoptosis, proliferation, differentiation, and cell behavior important for migration. Effects on these essential processes of brain cell development *in vitro* are predicted to translate into profound effects on *in vivo* brain morphology and function.

Our lab has recently utilized a commercially available neural progenitor cell line, ENStem-A™ as a model for conducting *in vitro* toxicological assessments (Shin, 2006). The hNPCs undergo differentiation upon withdrawal of growth factors. The cells are cultured for up to Day 21 post inducing differentiation and morphological changes and protein expression are compared in proliferating versus differentiating hNPCs.

In our immunofluorescence data of ENStem-A™ hNPC cultures, the neural progenitor cell marker Nestin expression decreased after 24 hrs, while the neuronal differentiation marker β -tubulin III increased over time in culture as is expected as neuronal differentiation proceeds. However this was inconsistent with our protein expression data from Western Blot which did not show this same decrease in Nestin over time. This could mean that cells are still transitioning and co-expressing both markers during the early weeks of differentiation or that cells in culture are differentiating at different times or rates.

NPCs have two essential properties of progenitor cells which are self-renewal (proliferation) and multipotency. Once cells commit to neural specific stages, self-renewal decreases. Therefore, while continuous proliferation of cells is observed in the early phases of differentiation, self-renewal (proliferation) decreases in differentiating cultures over time. PCNA protein expression reflects this change under differentiating cell culture conditions. PCNA is constantly expressed during the first week, then decreases its expression at later time points (Day 14, 21). Morphological observations also show continuous increase in cell number. The cells migrate to form networks and these get denser and bigger over time in culture. Formation

of the neural networks is an important indicator of neuronal differentiation and maturation that could provide an important model for developmental neurotoxicology.

These findings are then compared to our lab's previous mouse embryonic midbrain *in vitro* micromass culture and *in vivo* data bases readily available by the Allen Brain Institute. The Allen Brain Institute's developing mouse brain Atlas quantifies dynamic changes in expression of markers of differentiation over time and by specific brain regions. These data sets help to put our hNPC model in context. Initial comparisons indicate that our hNPCs differentiation culture system follows the basic pathway dynamics of this "gold standards" of brain development, thus confirms the significance of our culture system. Due to the complexity of these dynamic expression patterns, a more thorough characterization of each model will be necessary for meaningful comparisons.

It is important to note of species differences. In order to extrapolate results across biological levels, responses and particular relative responses will be needed. In several recent manuscripts, a common comparison of pathway response has been proposed, but not yet characterized for neuronal proliferation and differentiation. In order to extrapolate results across biological levels, responses and particular relative responses will be needed.

Expression of α -synuclein in more differentiated hNPCs suggests that differentiating hNPCs not only express neuronal structural markers but also undergo functional maturation. Characterization of which brain region and what types of cells our hNPCs are differentiating to is of interest. Expression of the presynaptic marker is an exciting observation because it indicates that the differentiating hNPCs are maturing to have functional roles. Because synaptic function is associated with neurotransmitter release, extensive characterization of neurotransmitter activity such as expression of glutamate receptors (NMDA, GluR1, GluR5), GABA, dopamine and serotonin in the differentiating hNPCs will provide clues about the specific functions taken on by differentiating neurons in our cultures system. Temporal changes in expression of forebrain (Otx1, Otx2, Foxg1, Pax6 and Tbr1), midbrain (EN2), and hindbrain (Gbx2, Egr2,

Hoxb3) markers will allow better understanding of which brain regions our ENStem-A™ hNPCs are differentiating to. Also, marker expression changes for glial fibrillary acidic protein (GFAP) evaluate the presence of astrocytes.

It is also important to be aware of the gender differences in brain development and neuronal differentiation processes. ENStem-A™ cells have an XX genotype and may fail to fully elucidate the dynamics of conditions like autism that disproportionately affect males (Wizemann 2001; McCarthy et al. 2012). Future development of hNPC models should utilize both female and male cell lines. Utilizing multiple cell lines with greater genetic diversity on diverse populations would better represent of the range of potential effects from chemical exposures.

Challenges with optimization of hNPC culture were that it was hard to get constant difference under proliferation verses differentiation cell culture conditions. After troubleshooting, we were able to optimize the hNPC culture system. Our plating density was 200,000 cells/mL which followed the recommendation from the commercial supplier. Interestingly, our commercial supplier of these cells has recently modified their cell plating density conditions. We are now interested in testing higher plating densities, micromass, and 3D culture of the hNPCs to find whether this would give stronger signals for differentiation.

Future studies of characterization of cell types under differentiation cell culture conditions may use confocal imaging to characterize cell by cell expression of these markers. Confocal imaging will help illuminate the process of differentiation in these cultures. Understanding these dynamics will help improving and stabilizing culture conditions to more accurately reflect *in vivo* developmental processes.

Part II. Effects of CP Exposure on hNPCs under Proliferation or Differentiation

Cell Culture Conditions

Results

Morphology

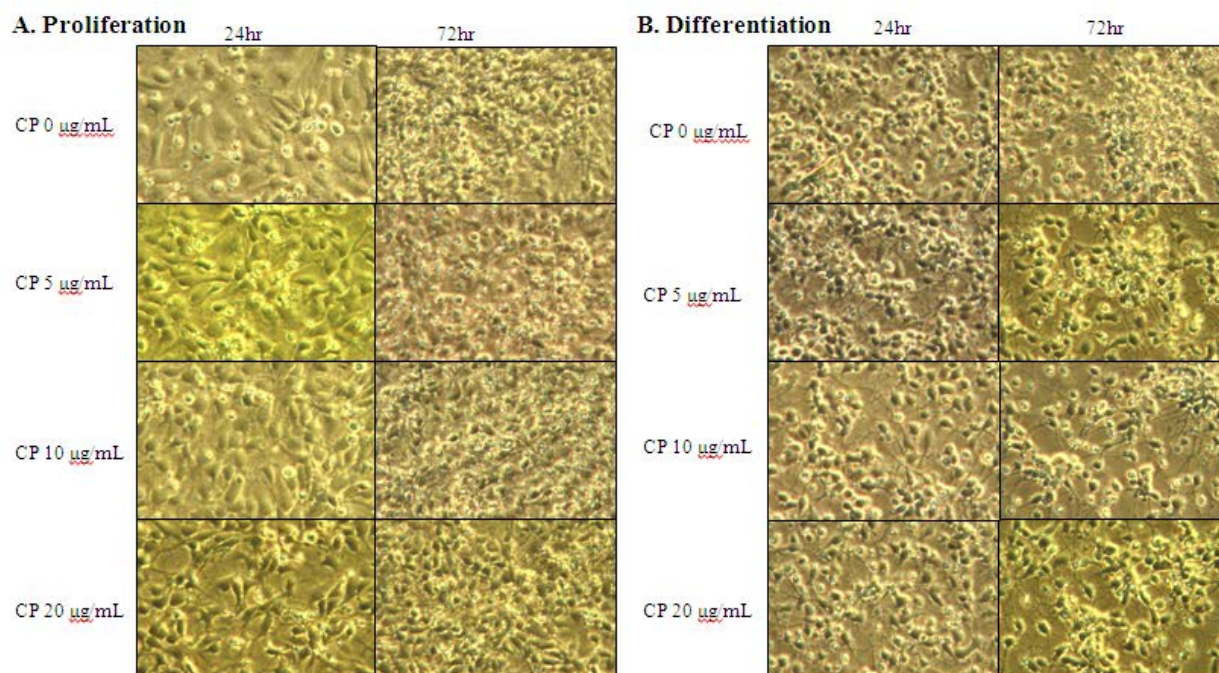


Figure 12 Phase-contrast morphology of proliferation or differentiation hNPCs at 24hrs and 72hrs after treatment with Chlorpyrifos (0-20 $\mu\text{g/mL}$)

Compared to 24hr, the cell numbers increase at 72hrs both in proliferation and differentiation conditions. With CP treatments, cell viability decreases more dramatically under differentiating cell culture conditions than the proliferating cell culture condition. All images are taken with same magnification (20x).

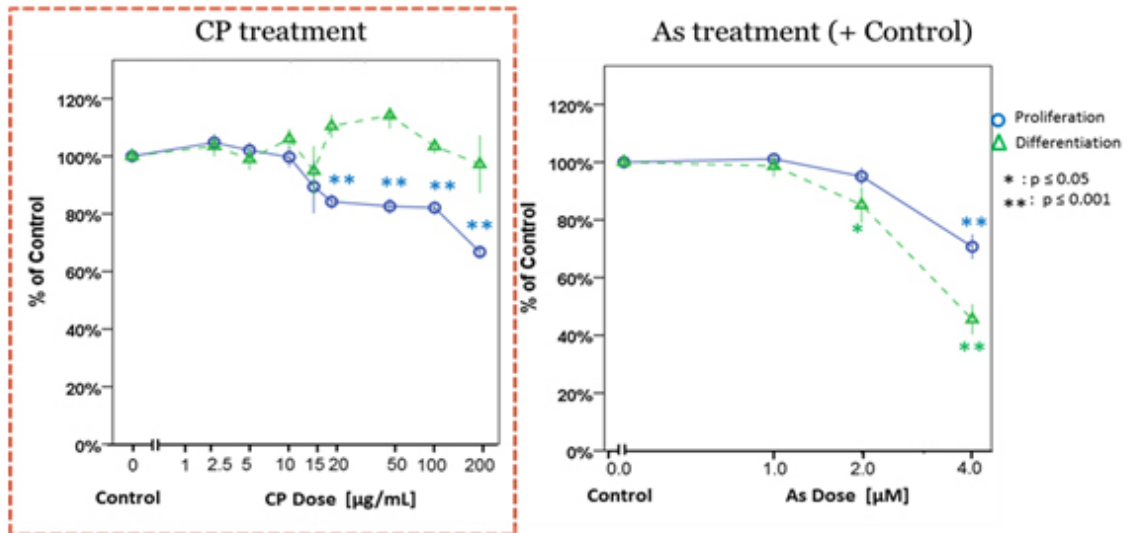
Morphologic observations show increases in cell population from 24 hrs to 72 hrs under both proliferation and differentiation cell culture conditions (Figure 12). The shape of cells under control (CP 0 $\mu\text{g/mL}$) proliferation cell culture conditions is constant over time. When cells started differentiation, the cells become more round and start to show neurite outgrowth. hNPCs were exposed to CP at the time of establishing either proliferation or differentiation conditions (24 hrs after plating with 200,000cells/mL density). Post treatment with CP concentrations 0-200 $\mu\text{g/mL}$, dose-dependent decrease in cell viability and morphological changes were observed at

24 hrs and 72 hrs. In CP treated differentiation conditions, disconnected neurite outgrowth and dose-dependent decreases in cell viability were observed.

Cell Viability Assays (Alamar Blue)

Figure 13 Alamar Blue cell viability assay with CP or As treatment in proliferation and differentiation hNPCs

A. 24 hrs



B. 72 hrs

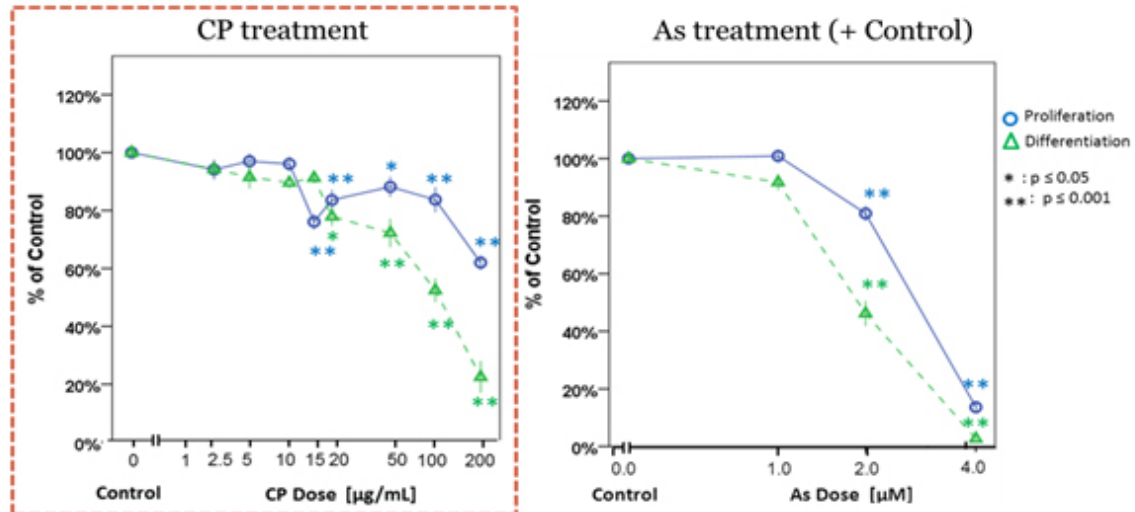


Figure 13 Alamar Blue cell viability assay with CP or As of hNPCs under proliferation or differentiation culture conditions

Cells were exposed to chlorpyrifos (CP 0-200 $\mu\text{g}/\text{mL}$) or arsenic (As 0-4 μM) under proliferating (Blue) and differentiating (Green) cell conditions for hNPCs at A. 24hrs and B. 72hrs. Data are reported as percent of the controls \pm SE. For CP treatment, there were significant differences with a $p \leq 0.001$ between the proliferating and differentiating cell culture conditions at both 24hrs and 72hrs using a mixed effect model with experiment preparation dates as a random effect. Statistical analysis was also done using One Way ANOVA within each cell culture condition across doses. Significant decreases were seen with $p \leq 0.001$ for 24hr proliferation, 72hr proliferation, and 72hr differentiation. No significant changes were found for CP treatment at 24hr under differentiating cell culture conditions ($p \geq 0.05$).

Arsenic was used as a positive control. Significance in response to As exposure ($p = 0.001$ and $p \leq 0.001$) was seen for differences between cell under the proliferating and differentiating cell conditions at both 24hrs and 72hrs post treatment, respectively, using a mixed effect model. One Way ANOVA analysis of dose response for each cell culture condition had significant decrease with $p \leq 0.001$ for both proliferating and differentiating cell culture conditions at both time points.

* indicates significance at $p \leq 0.05$ and ** indicates significance at $p \leq 0.001$ of treatment based on Dunnett t-tests compared to controls. Data is representative of at least three independent experiments.

Alamar blue assay measurements of cell viability reveal that proliferating and differentiating cultures respond to CP and As treatment with different degrees of sensitivity (Figure 13). Dose-dependent decreases of cell viability of hNPCs are observed in the proliferating cells after 24 hrs treatment, no significant changes are seen in the differentiation culture. However, after 72 hours, a significant dose-dependent decrease in cell viability of hNPCs under differentiation cell culture condition is observed. Dose-dependent decreases in response to CP were significantly greater on cells under differentiation cell culture condition. At CP concentration of 20 $\mu\text{g}/\text{mL}$, cell viability of differentiation is less ($78\% \pm 3.2\%$) than in the proliferation ($84\% \pm 3.6\%$) hNPCs. This suggests that differentiating neuronal cells are more sensitive to CP treatments than proliferating cells after 72 hours of treatment. The same sensitivity difference was seen with As in two cell culture systems.

Cell Viability Assays (Live and Dead Cell Staining)

The Live and Dead Cell Staining assay, also called 3 color assay, was used to visualize the live cells (green, Calcein AM), dead cells (red, Propidium Iodide), and nucleus (Blue, Hoechst 33342). The total number of nuclei from 3 color assay images was counted using Image J software (National Institute of Health, Maryland, USA) and the percent changes relative to 24 hrs controls of each cell type are shown in Figure 14. For CP treatment, there is a significant difference cell response between the proliferating and differentiating hNPCs at both time points evaluated (24 hrs $p \leq 0.001$ and 72 hrs $p = 0.008$). Within each cell culture condition, significant dose-dependent decrease in cell number is observed in both proliferation and differentiation cell culture conditions (at both time points evaluated (24 hrs, 72 hrs) ($p \leq 0.001$). CP affects both proliferation and differentiation conditions in a similar dose-related manner. However, significant effects are observed at lower doses in differentiating cells. At 24 hrs post treatment, we observe significant decreases in cell number following exposure to 10 $\mu\text{g}/\text{mL}$ of CP during differentiation, while 50 $\mu\text{g}/\text{mL}$ is the lowest dose with a significant effect on cell number in proliferating cultures. At 72 hrs post treatment, significant decrease in cell number is seen at 10 $\mu\text{g}/\text{mL}$ of CP during differentiation and at 20 $\mu\text{g}/\text{mL}$ of CP during proliferation (Figure 14). This suggests that the percent change of total cell number is decreasing with more sensitivity in differentiating conditions.

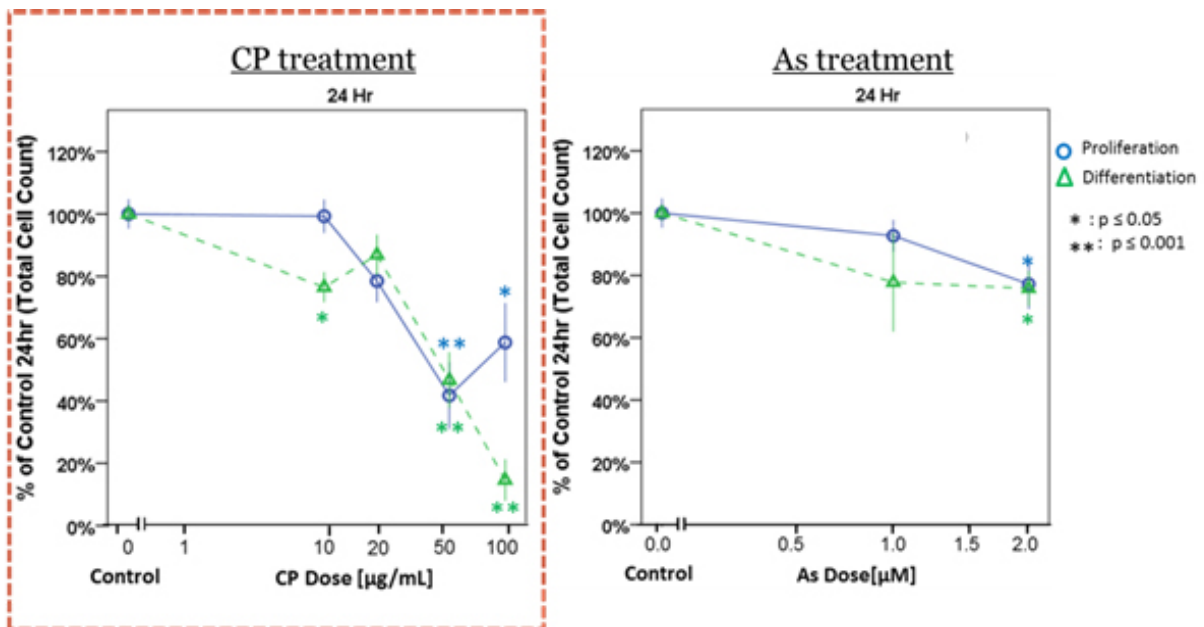
Comparing proliferation and differentiation at 24 hrs, cell viability is decreased by a similar rate up to CP 50 $\mu\text{g}/\text{mL}$ (% live cells are 56% under proliferation conditions and 58% under differentiation conditions). At CP 100 $\mu\text{g}/\text{mL}$, proliferating cells demonstrate higher cell viability (68%) whereas differentiation continued with its decreasing trend (37%). At 72 hrs, there is an even more dramatic decrease in percent cell viability in both proliferation and differentiation. Differentiating cells gradually decrease in viability as the CP concentration increases, with only 37% cell viability at CP 100 $\mu\text{g}/\text{mL}$. 35% of proliferating cells are viable at CP 50 $\mu\text{g}/\text{mL}$ while at CP 100 $\mu\text{g}/\text{mL}$ there is an increase in cell viability (55%).

Arsenic, the positive control, agreed to show decreases in cell viability at 24 hrs and 72 hrs in both culture conditions. There is significant difference cell response between the proliferating and differentiating hNPCs at 72 hrs ($p \leq 0.001$) but not at 24 hrs ($p = 0.668$). However, the dose-response within proliferation or differentiation cell culture conditions have significance decrease at both time points ($p \leq 0.05$ for 24 hrs proliferation, 24 hrs differentiation, and 72hr proliferation; $p \leq 0.001$ for 72hr differentiation). The changes are dramatic at 72 hrs and more toxic during differentiation.

Figure 15 shows the images of 3 color assay. The images were all captured at 20x magnification. From top to bottom represents changes over time, and from left to right represents changes over CP concentrations. Looking at the non-treated cells in both conditions, cells increase in their cell population. Once the cells fill up the dish, proliferating cells squeeze in tightly and differentiating cells form in clusters like they are starting to form neurospheres. In both non-treated cell conditions, increased numbers of dead cells (PI staining) are observed along with noticeable increases of live cells. While neurite outgrowth is observed in differentiation hNPCs, this morphological change is not seen in proliferation hNPCs. CP treatments induces disconnected neurite out-growths and less dense neurosphere formations in differentiating cells, suggesting exposures to CP in differentiating cells may cause more significant morphological changes than in proliferating cells.

Figure 14 Total number of nucleus changes with CP or As treatment in 3 color assay in proliferation and differentiation

A. 24hrs



B. 72hrs

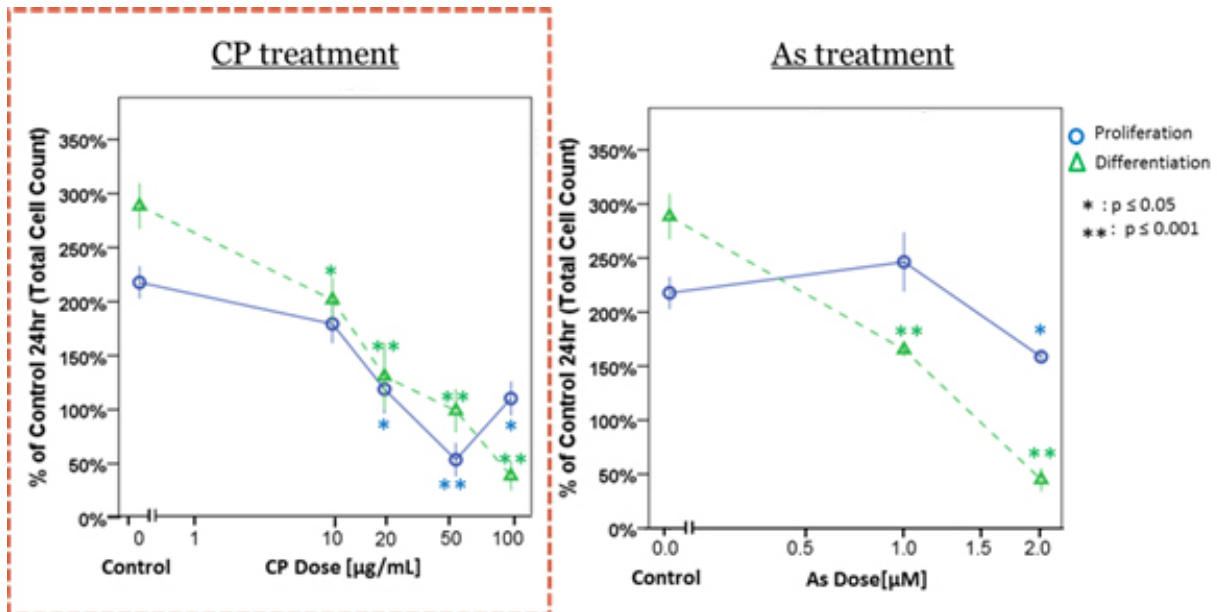


Figure 14 Total number of nucleus changes with CP or As treatment using 3 color assay analysis in hNPCs cultured under proliferation and differentiation conditions

Total number of nucleus were counted using Hoechst 33342 from 3 color assay. Cells were exposed to CP (0-100 $\mu\text{g}/\text{mL}$) or As (0-2 μM) under proliferating (Blue) and differentiating (Green) cell conditions for hNPCs at A. 24hrs and B. 72hrs. Data are reported as percent of the 24hr controls \pm SE. For CP treatment, there was a significant difference $p \leq 0.001$ and $p=0.008$ between the proliferating and differentiating cell culture conditions at both 24hrs and 72hrs, respectively, using a mixed effect model with experiment preparation date as a random effect. Statistical analysis was also done using One Way ANOVA within each cell culture condition across doses. Significant decreases with $p \leq 0.001$ were seen for both cell culture conditions at both time points.

Arsenic was used as a positive control. There was no significant difference in As response seen between hNPCs under proliferation and differentiation cell culture condition at 24hr ($p \geq 0.05$). Significance with a $p \leq 0.001$ was seen for differences between cells under the proliferating and differentiating cell conditions at 72hrs. One Way ANOVA analysis of dose response for each cell culture condition had significant decreases for proliferating ($p=0.045$) and differentiating ($p=0.047$) cell culture conditions at 24hrs. For 72hrs, significant decreases were seen in both cell culture conditions, proliferation ($p=0.020$) and differentiation ($p \leq 0.001$).

* indicates significance at $p \leq 0.05$ and ** indicates significance at $p \leq 0.001$ of treatment based on Dunnett t-tests compared to controls. Data is representative of at least three independent experiments.

Figure 15 Illustration of live, dead and nucleus of proliferation or differentiation hNPCs with or without CP (0-100 µg/mL) for 24-72hrs

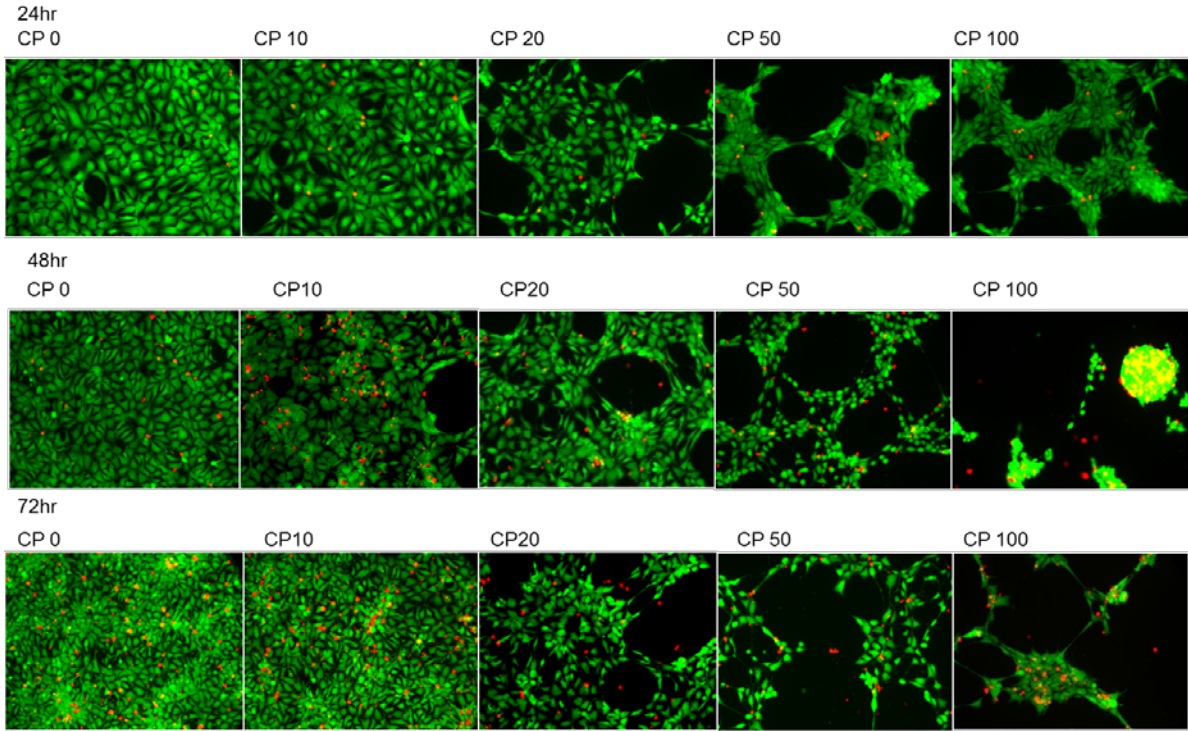
3 color assay.

Green : CalceinAM (Live cell)

Red : Propidium iodide (dead cell)

Blue : Hoescht 33342 (Nucleau)

A1. Proliferation – Live and Dead Cells



A2. Proliferation - Nucleus

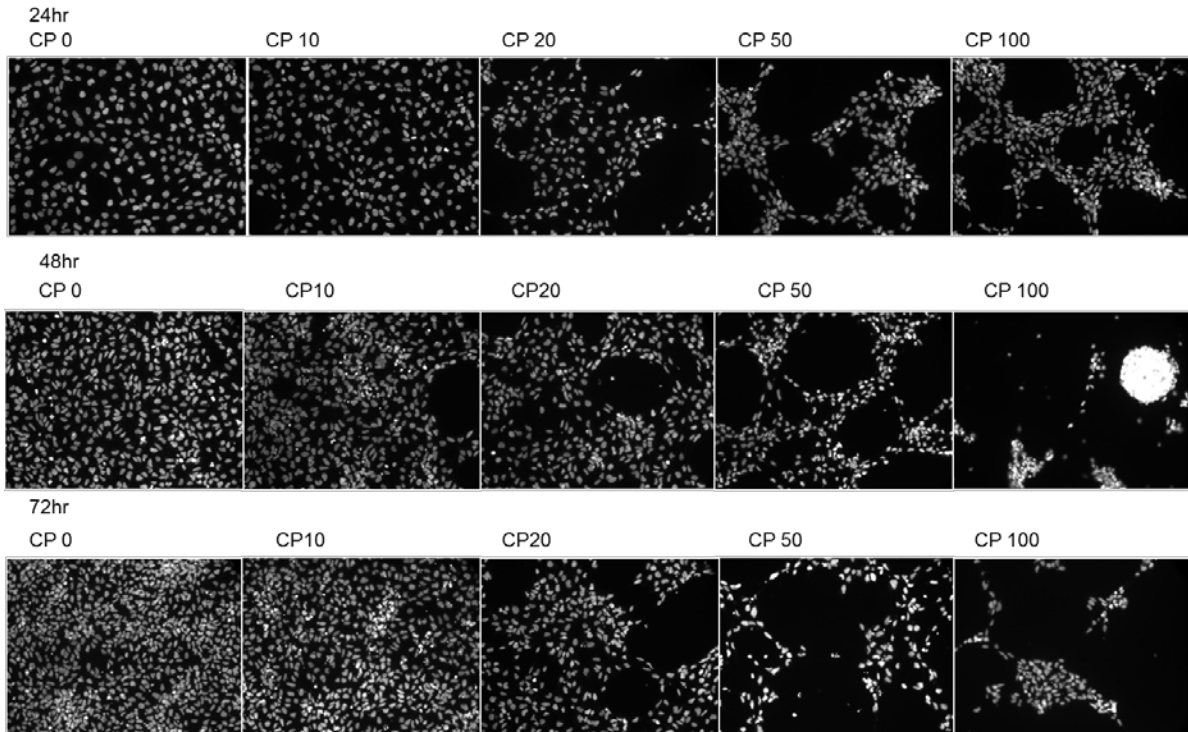
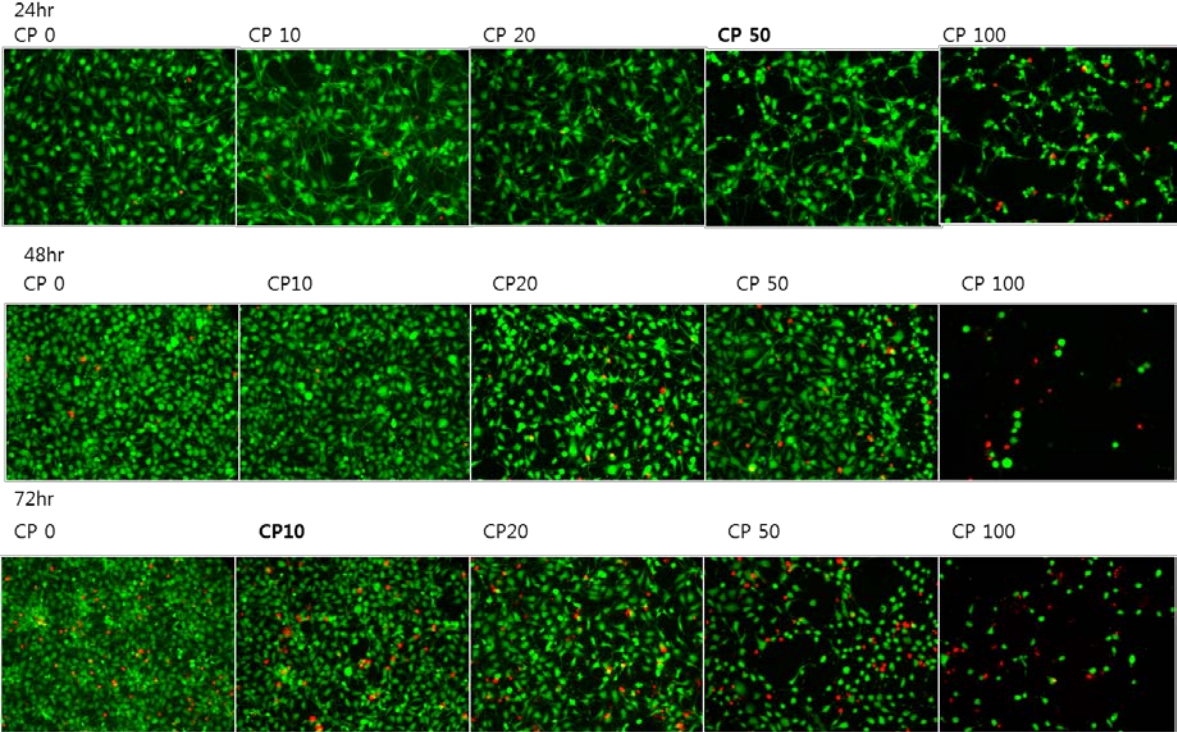


Figure 15 Illustration of live, dead and nucleus of proliferation or differentiation hNPCs with or without CP (0-100 µg/mL) for 24-72hrs (continued)

3 color assay.
 Green : Calciin AM (Live cell)
 Red : Propidium iodide (dead cell)
 Blue : Hoescht 33342 (Nucleau)

B1. Differentiation – Live and Dead Cells



B2. Differentiation - Nucleus

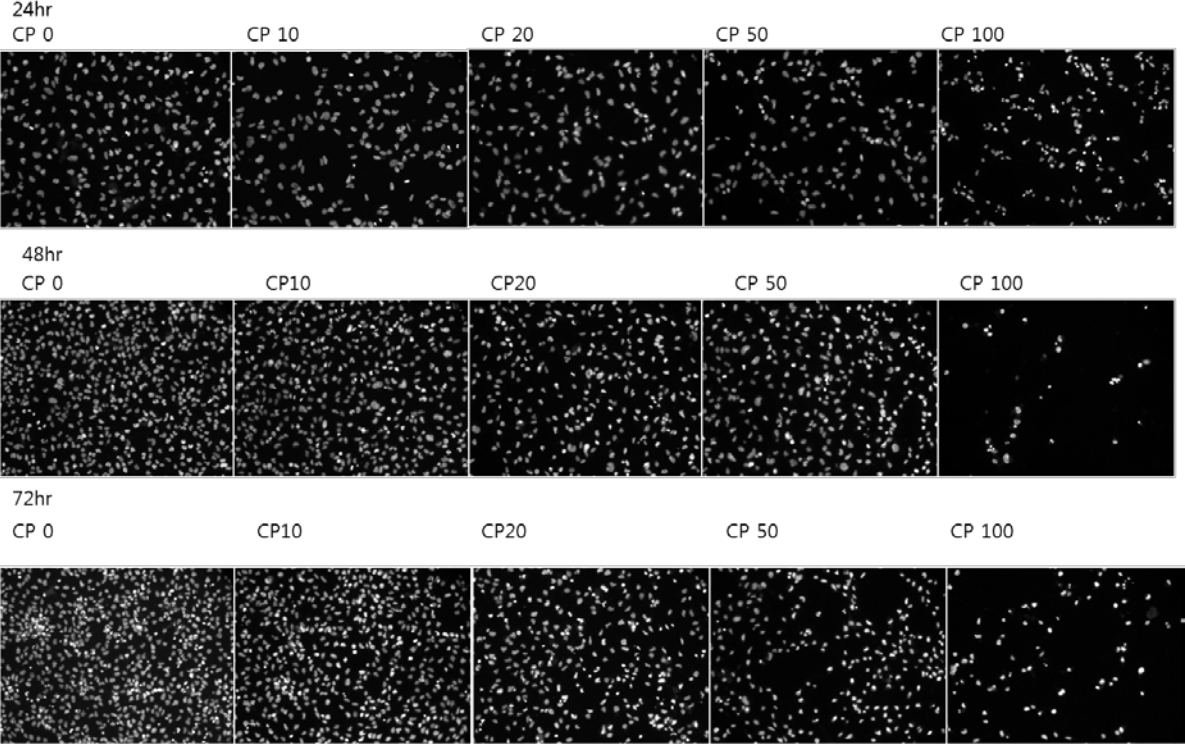


Figure 15 Illustration of live, dead and nucleus of proliferation or differentiation hNPCs with or without CP (0-100 µg/mL) for 24-72hrs

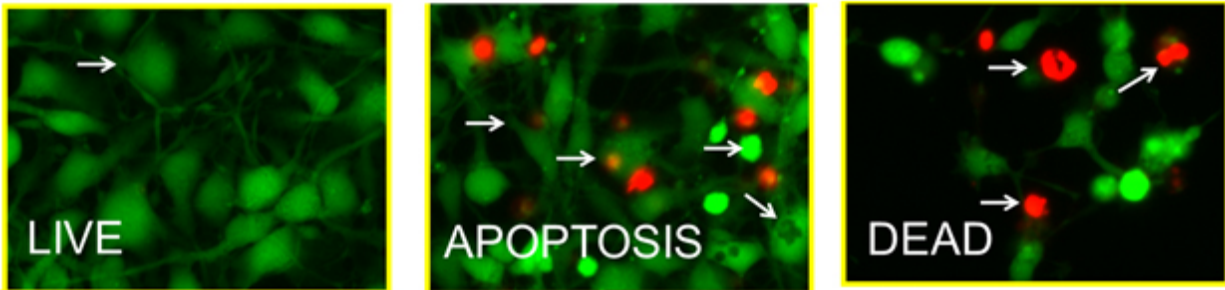
Calcein AM shown in green for live cell, and propidium iodide (PI) shown in red for dead cells. Hoechst 33342 for total nucleus in blue is used to count the total cell number. To better view the nucleus, the nucleus in the images are shown in white. Exposure to Chlorpyrifos (CP) induced dose-dependent decreases of cell viability in both proliferation and differentiation culture condition. Images are representative of at least three independent experiments.

The total cell number was counted using the nucleus staining (Hoechst 33342) and the results are shown in Figure 14. Among the total cells, the live, dead, and apoptotic cells were characterized as described in Figure 5 and counted manually using Image J software (National Institute of Health, Maryland, USA) to evaluate the percent of live, dead, and apoptotic cell population among CP treated and non-treated in proliferating or differentiating cultures. Examples of healthy live cell, dead cell, and apoptosis (injured) cells are shown on the top of Figure 15. Cells with evenly stained Calcein AM are healthy live cells and cells stained with PI are dead cells. When cells dye through apoptosis, condensation and fragmentation of nuclei is commonly observed. We decided that when the fragments of PI stains are overlapping or located close by, those are counted as one dead cell. Besides live and dead cells, there are cells that are going through apoptosis process. Cases include having PI stains inside Calcein AM stained live cell. PI can only pass through disrupted cell membrane. Therefore, PI stains in green live cells indicate that the cell is going through a programmed cell-death pathway. Also, cells that are stained with Calcein AM, but have enlarged vesicles or extremely bright fluorescence with condensed cell shapes, are considered as apoptotic cells.

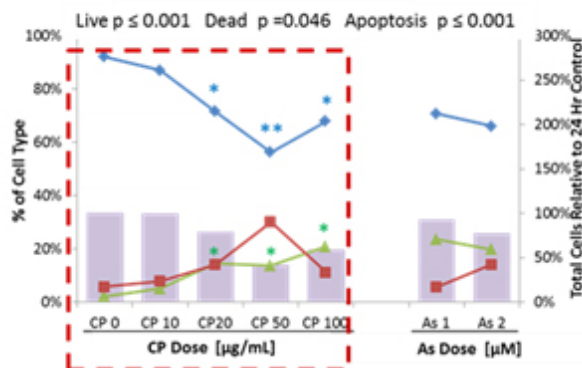
Manual counts of live and dead cells reveal significant decreases in the percent of live cells with increasing CP and As doses (Figure 16). CP induced death was associated with apoptosis because nucleus condensation and fragmentation were typical morphology for cell death induced by apoptosis process. Although the percent of dead cells is fairly constant

between proliferation and differentiation, apoptotic cells are much more dramatically increased by CP exposure in differentiating cells.

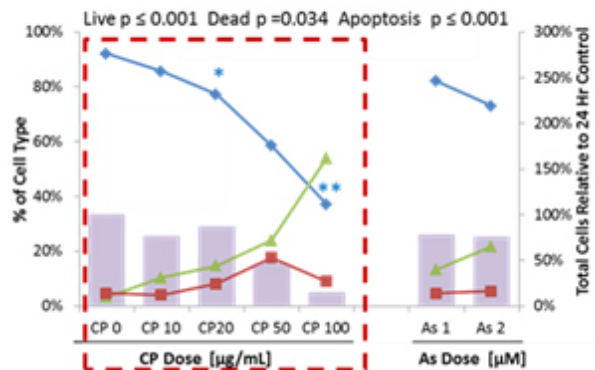
Figure 16 Percent of live, dead, and apoptotic cells of CP treated proliferation and differentiation hNPCs



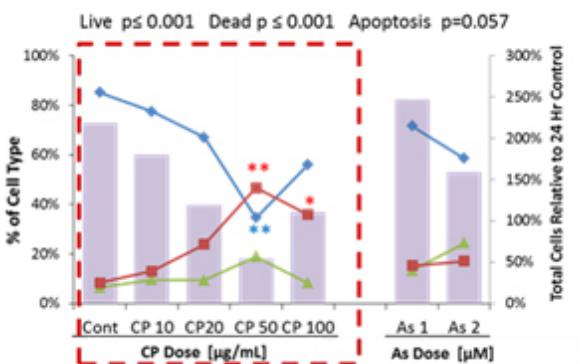
(A) 24hr Proliferation



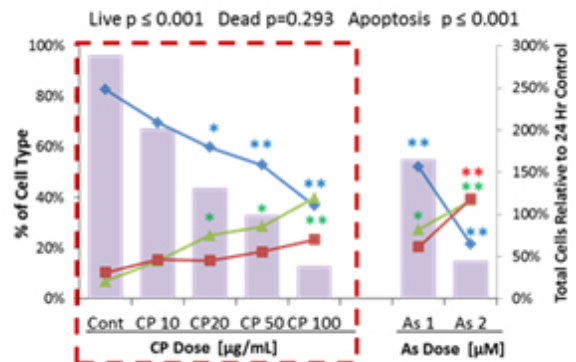
(B) 24hr Differentiation



(C) 72hr Proliferation



(D) 72hr Differentiation



■ Total Cells ● Live ▲ Apoptosis ■ Dead

Figure 16 Percent of live, dead, and apoptotic hNPCs following CP treatment under proliferation and differentiation cell culture conditions

Images on top are the examples of healthy live cells (Calcein AM, Green), apoptotic cells, and dead cells (PI, Red) from our cultures. The percent of live, dead, and apoptotic cells among the total attached cells in each CP dose-treatment are plotted on the graphs (A-D). The lines indicate percent of live, apoptosis and dead cells plotted on the left y-axis. The right y-axis refers to the gray bar graphs plotted for the total number of attached cells as a percent increase following 24hrs control for proliferation (A, C) or differentiation (B, D) cell culture conditions. Overall a dose-dependent decrease in live cells and increase in dead and apoptotic cells were seen with CP and As treatment under both cell culture conditions and two times of evaluation. Statistical analysis was done between doses using one way ANOVA across cell culture conditions.

* indicates significance at $p \leq 0.05$ and ** indicates significance at $p \leq 0.001$ of treatment based on Dunnett t-tests compared to controls. Data is representative of at least three independent experiments.

Significant differences were seen in hNPCs at 24hr following CP under differentiation and at 72hr following exposure under proliferation conditions where data are analyzed on percent of dead vs apoptotic cells ($p=0.010$, $p \leq 0.001$, respectively) using a mixed effect model (experiment preparation date as a random effect). For arsenic, hNPCs at 24hr post treatment in proliferation and differentiation cell culture conditions had significant difference for the percent of dead vs apoptotic cells ($p=0.011$, $p \leq 0.001$, respectively). No significant differences were seen for CP treatment on 24hr proliferation, 72hr differentiation and As treatment on 72hr proliferation and differentiation cell culture condition ($p \geq 0.05$).

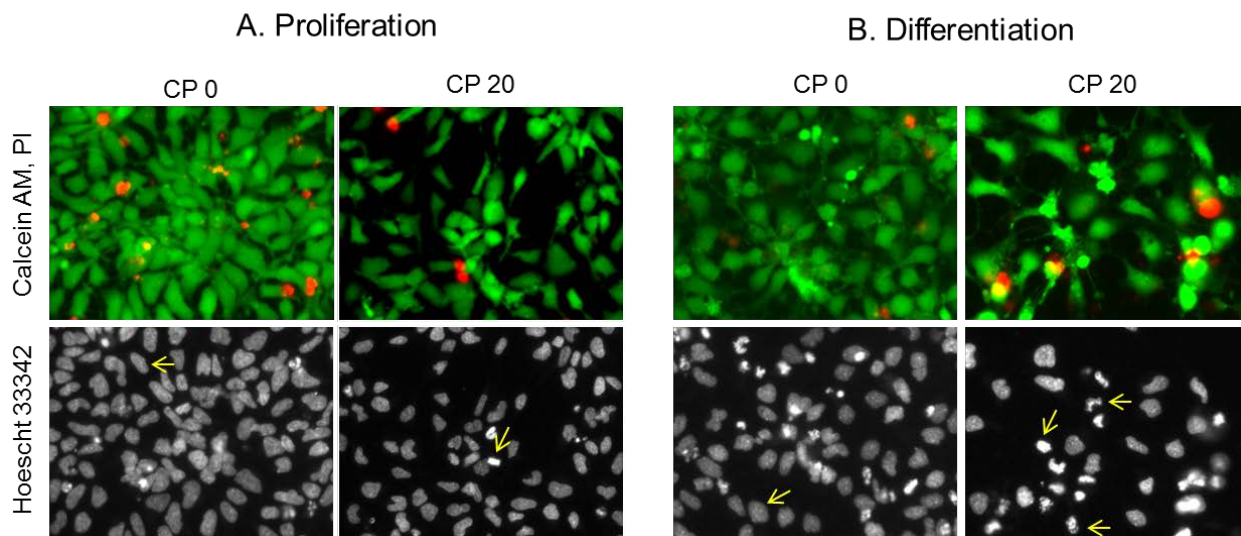


Figure 17 A closer view of 3 color assay with and without CP treatment (20 µg/mL). Top images are the live cells (Calcein AM, Green) and dead cells (PI, Red). Bottom images are nucleus (Hoescht 33342, White). Arrow in CP0 µg/mL demonstrates healthy nuclei, and arrow in CP20 µg/mL demonstrates apoptotic nuclei that are either condensed or fragmented.

Live and dead cell staining (3 color assay) shows the differences in cell morphology between proliferation and differentiation hNPC culture conditions. Calcein AM (green) stain shows disrupted neurite outgrowth (Figure 17. B. Differentiation, CP20 $\mu\text{g}/\text{mL}$). The morphological characteristic of apoptotic cells is that the nucleus gets condensed and fragmented. In CP 0 $\mu\text{g}/\text{mL}$ and 20 $\mu\text{g}/\text{mL}$ treated culture has cells with these characteristics. Apoptosis is a seen in normal cells, but with stress on cells, increased apoptosis and cell death are observed. Apoptotic cells are more frequently detected in hNPCs cultured under differentiation cell culture conditions than in cells cultured under proliferation cell culture conditions (Figure 16, Figure 17).

Immunofluorescence

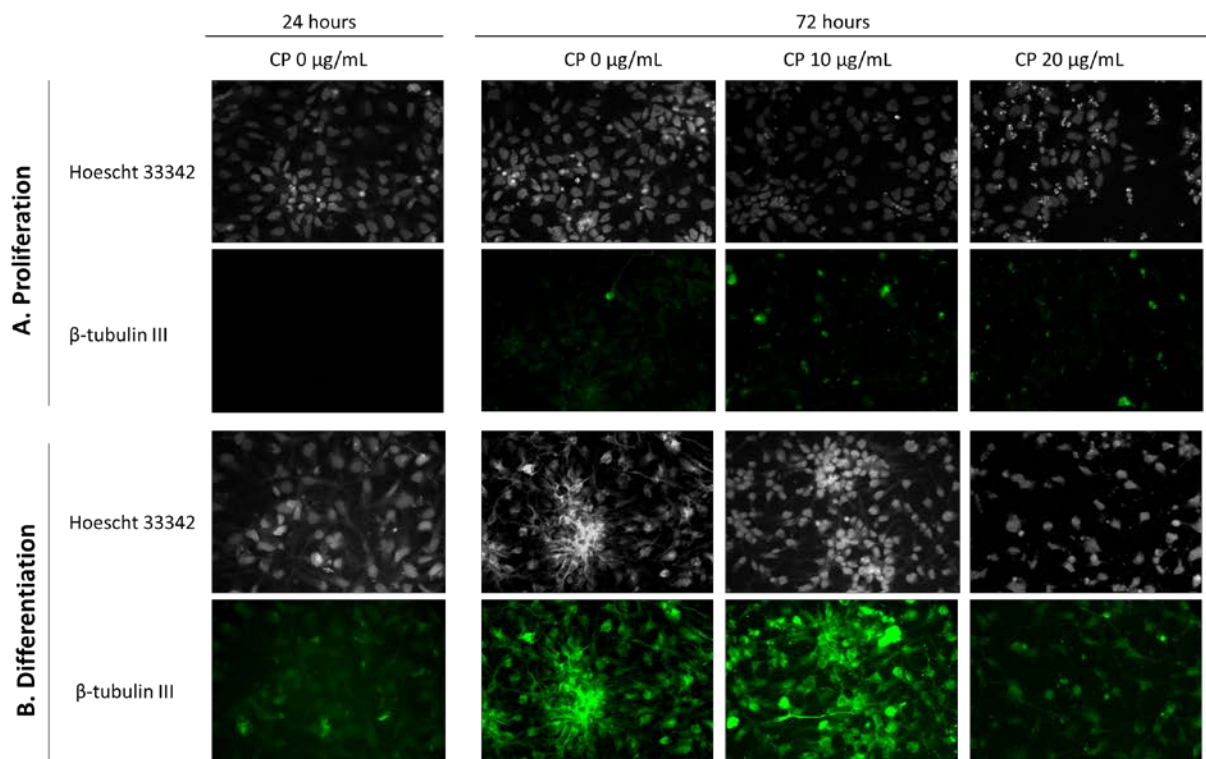


Figure 18 Immunofluorescence expression levels of β -tubulin III on CP treated hNPCs during proliferation and differentiation

From the left represents untreated 24hr cells followed by 72hr CP treatment with 0-20 $\mu\text{g}/\text{mL}$. Top shows the total nucleus using Hoechst 33342, and bottom shows the expression of β -tubulin III. A. β -tubulin III was expressed less in proliferating cells compared to differentiating cells. B. In differentiating hNPCs, β -tubulin III had increase in expression at 72hrs compared to 24hrs. Untreated cells at 72hr started to migrate and initiated network formation. Dose-dependent decrease in cell number, network formation was observed. Cells were less intensely stained at CP 20 $\mu\text{g}/\text{mL}$.

Western Blot

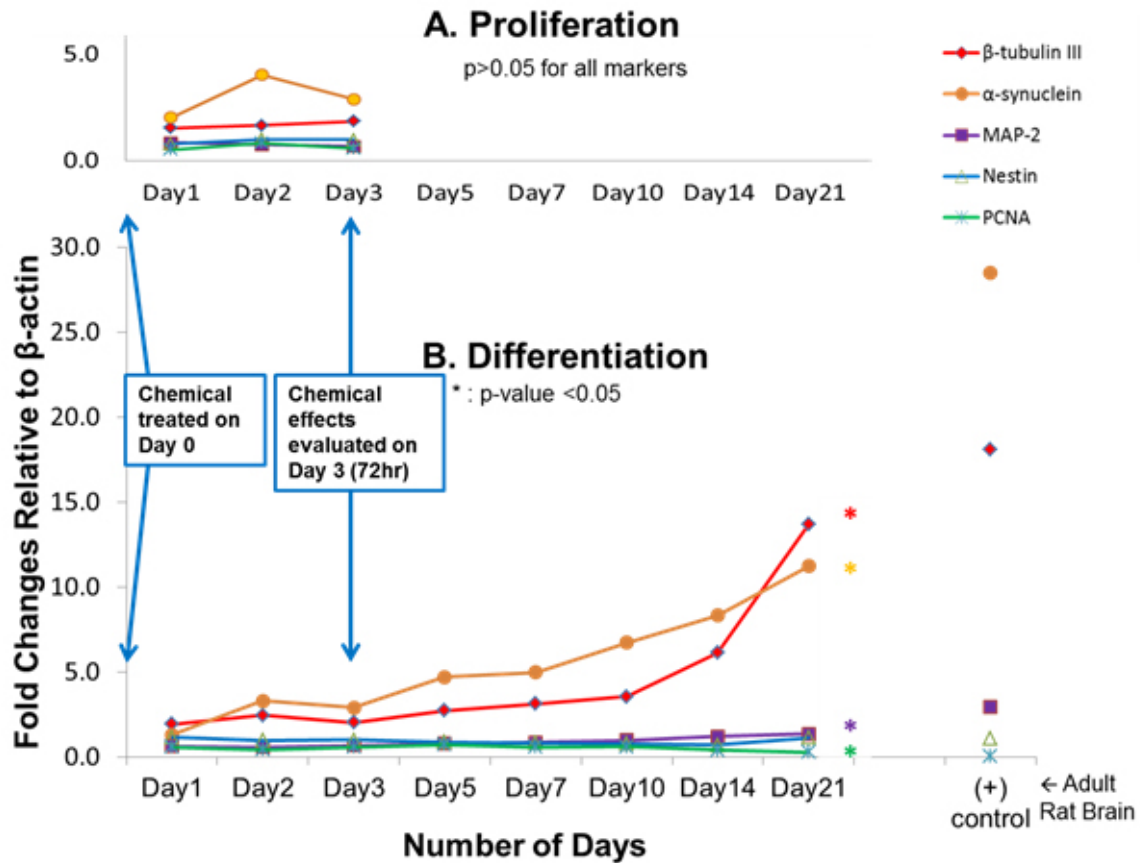


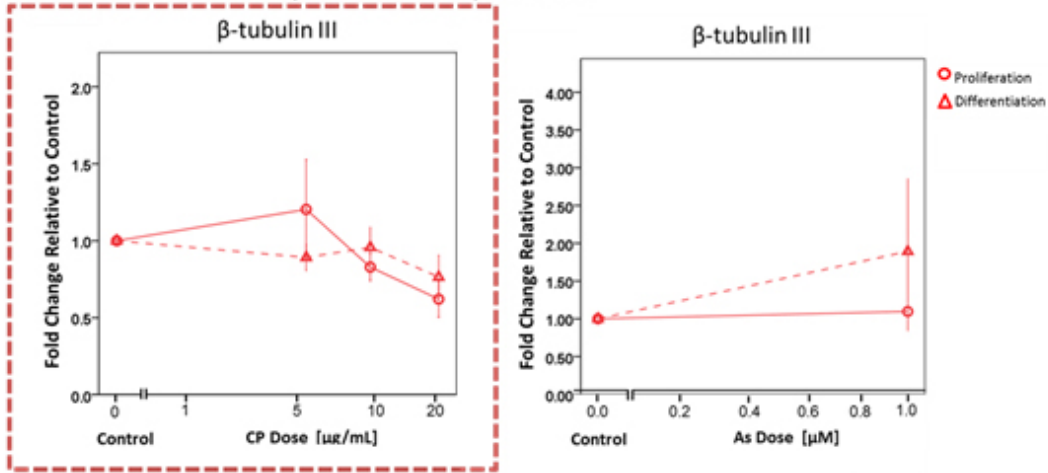
Figure 19 Characterization of protein expression markers in hNPCs over time and under proliferating and differentiating cell culture conditions

hNPC cells were cultured under proliferation and differentiation cell culture conditions to examine culture dynamics. Culture conditions are described in page 15-17 and in Figure 3. By Day 3, the cells reach their full confluency in both untreated proliferating and differentiating hNPCs. Neural specific stage markers start to express at this time point (day 3), Therefore, we examined the effects of CP or As treatment at 24 hrs and 72 hrs post Day 0 treatment. One way ANOVA was run comparing fold changes between days in culture. Only differentiating cell culture condition had significant changes over time for B-tubulin III, α -synuclein, MAP-2, and PCNA. Nestin did not change over time under either culture condition.

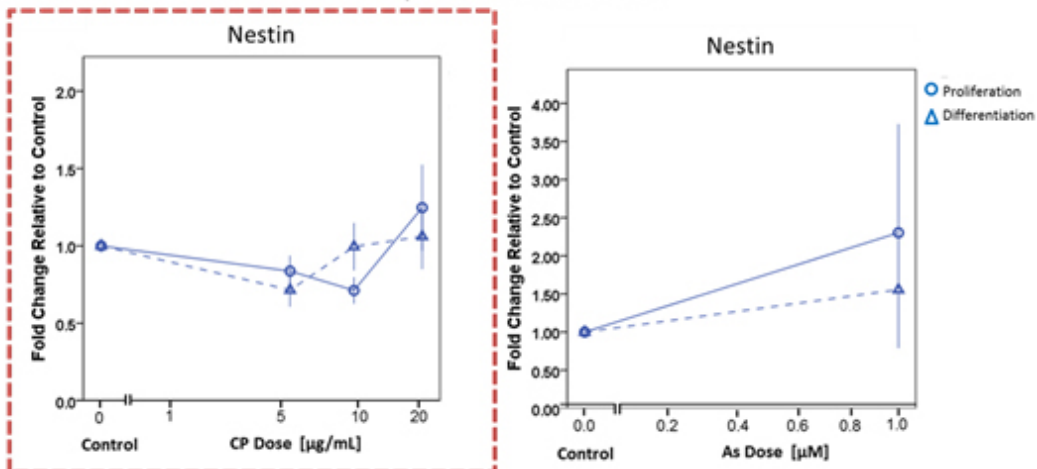
* indicates significance change ($p < 0.05$) in neuronal marker expression across time.

Figure 20 Changes in neuronal stage marker expressions with CP or As treatment in 72 hrs of proliferation and differentiation

A. Neuronal differentiation protein marker: β -tubulin III



C. Immature neuronal differentiation protein marker: Nestin



B. Neuronal differentiation protein marker: Microtubule Associated Protein 2 (MAP2)

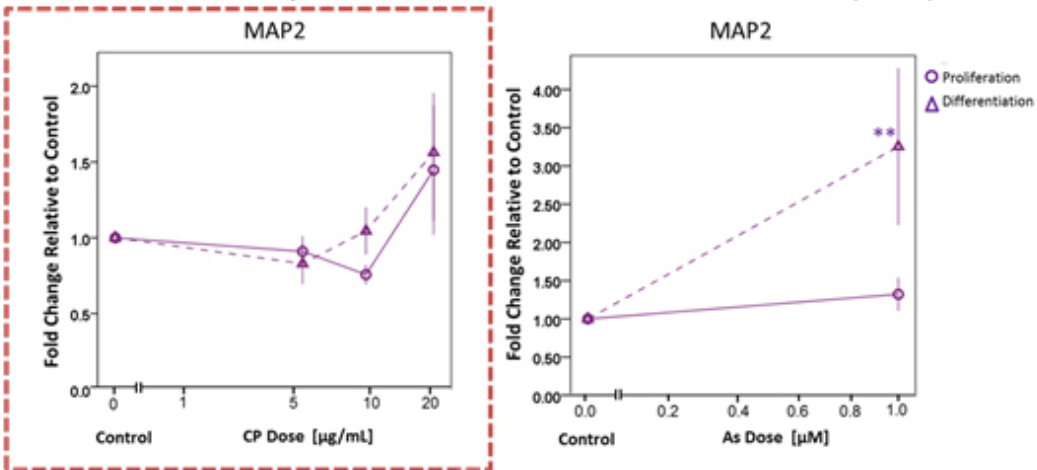
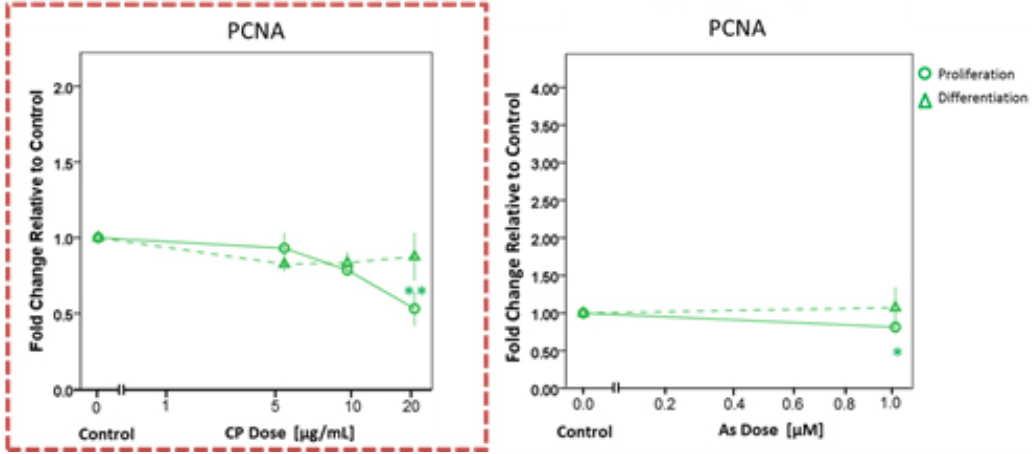
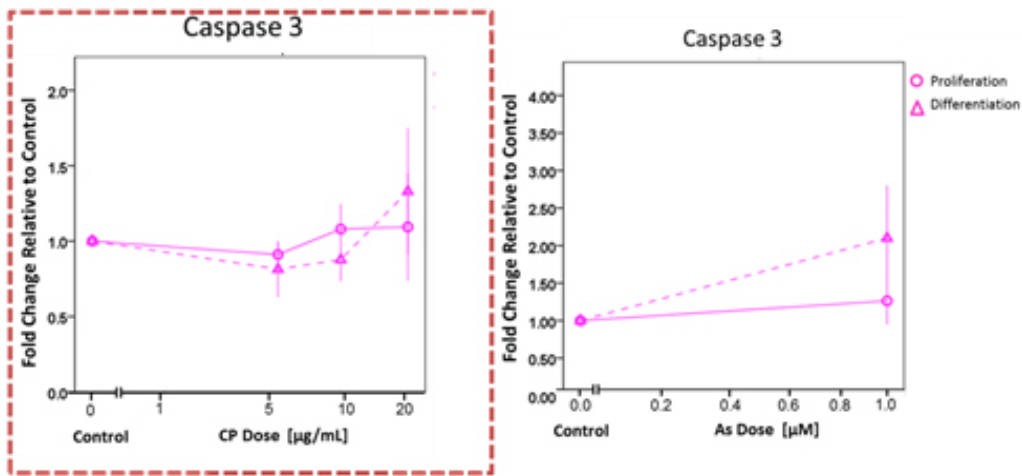


Figure 20 Changes in neuronal stage marker expressions with CP or As treatment in 72hrs of proliferation and differentiation (*continued*)

D. Proliferating protein marker: Proliferating Cell Nuclear Antigen (PCNA)



E. Apoptosis protein marker: Caspase 3



F. Stress Protein marker : Mitogen-Activated Protein Kinase p42 and p44 (pMAPK p42/44)

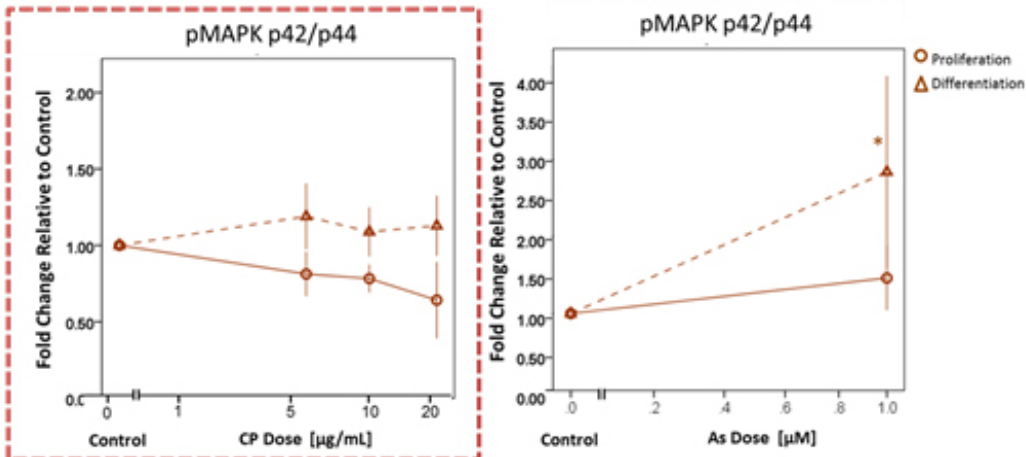


Figure 20 Changes in neuronal and stage specific protein marker expressions following CP or As treatment of hNPCs cultured under proliferation and differentiation conditions at 72 hrs post treatment

Protein expression levels for neuronal and stage specific markers in hNPCs grown under proliferation (line) and differentiation (dotted line) cell culture conditions. Cells were examined after 72 hours of CP (0-20 $\mu\text{g}/\text{mL}$) or As (1 μM) treatment. In this set of figures, neuronal differentiation protein markers included β -tubulin III (A) and Microtubule Associated Protein 2 (MAP2) (B). Immature neuronal differentiation protein marker - Nestin (C), proliferating protein marker – Proliferating Cell Nuclear Antigen (PCNA) (D), apoptosis protein marker - Caspase 3 (E), and stress marker – Mitogen-Activated Protein Kinase p42 and p44 (pMAPK p42/p44) (F). β -actin was used as internal control and graphs represent the fold changes in protein relative to controls. Data is reported as fold changes of the controls \pm SE and represent at least three independent experiments. A mixed effect model analysis was conducted to identify significant differences between cell culture conditions and doses, using experiment preparation dates as a random effect. Arsenic was run as a positive control.

A: Neuronal differentiation protein marker, β -tubulin III, was analyzed using a mixed effect model and results showed a significant decrease in protein following CP exposure ($p=0.022$) but no detectable difference between the responses of this protein to CP exposure between hNPCs in proliferation vs differentiation cell culture conditions ($p\geq 0.05$). For arsenic, there was no significant effect of As treatment of hNPCs under any conditions ($p\geq 0.05$). No dose-dependent differences in β -tubulin III protein levels were observed following either CP or As treatment under either culture condition (One Way ANOVA and Dunnett t-tests $p\geq 0.05$).

B: Neuronal differentiation protein marker, MAP2, was analyzed using a mixed effect model and results showed a significant increase in protein following CP exposure ($p=0.044$) but no detectable difference between the responses of the protein to CP exposure between hNPCs in proliferation vs differentiation cell culture condition ($p\geq 0.05$). For arsenic, there was significant increase effect of As treatment of hNPCs ($p=0.0001$) and for the interaction between proliferation vs differentiation cell culture conditions ($p=0.002$). Differences in MAP2 protein levels were observed following As treatment under differentiating cell culture conditions (One Way ANOVA $p=0.001$).

C: Immature neuronal differentiation protein marker, Nestin, was analyzed using mixed effect model and results showed no significant changes in protein following CP or As exposure. No detectable difference between the responses of this protein to CP or As exposure between hNPCs in proliferation vs differentiation cell culture conditions were shown ($p\geq 0.05$). No dose-dependent significance ($p\geq 0.05$) was found in One Way ANOVA and based on Dunnett t-tests under both CP and As treatment.

D: Proliferating protein marker, PCNA was analyzed using a mixed effect model and results showed significant decrease in protein following CP exposure ($p=0.021$) but no detectable difference between the responses of this protein to CP exposure between hNPCs in proliferation vs differentiation cell culture conditions ($p\geq 0.05$). Dose-dependent decreases in PCNA protein level were observed following CP treatment under proliferating cell culture conditions (One Way ANOVA $p=0.001$), with a significant decrease under CP 20 $\mu\text{g}/\text{mL}$ (Dunnett t-tests $p\leq 0.001$). For arsenic, there was no significant effect of As treatment of hNPCs under any conditions ($p\geq 0.05$). Decrease in PCNA protein level was observed following As treatment under proliferating cell culture conditions (One Way ANOVA ($p=0.012$).

Figure 20 Changes in neuronal stage marker expressions with CP or As treatment in 72hrs of proliferation and differentiation (*continued*)

E: Apoptosis protein marker, Caspase 3, was analyzed using a mixed effect model and results showed no significant increase in protein following CP exposure ($p \geq 0.05$) and no detectable difference between the responses of this protein to CP exposure between hNPCs in proliferation vs differentiation cell culture condition ($p \geq 0.05$). For arsenic, the result showed a significant effect of As treatment of hNPCs ($p = 0.041$), but no detectable difference between hNPCs in proliferation vs differentiation cell culture condition ($p \geq 0.05$). No dose-dependent differences in Caspase 3 protein levels were observed following either CP or As treatment under either culture condition (One Way ANOVA, Dunnett t-tests ($p \geq 0.05$)).

F: Stress marker, pMAPK p42/p44, was analyzed using a mixed effect model and results showed no significant effect in protein following CP exposure ($p \geq 0.05$) and no detectable difference between the responses of this protein to CP exposure between hNPCs in proliferation vs differentiation cell culture condition ($p \geq 0.05$). For arsenic, the result showed a significant effect of As treatment of hNPCs ($p = 0.037$), but no detectable difference between hNPCs in proliferation vs differentiation cell culture condition ($p \geq 0.05$). Increase in pMAPK p42/p44 was observed following As treatment under differentiating cell culture condition (One Way ANOVA $p = 0.033$).

In the graphs, * indicates significance at $p \leq 0.05$ and ** indicates significance at $p \leq 0.001$ based on Dunnett t-test compared to controls. Data is representative of at least three independent experiments. Note that the y-axis scale is different between CP (2.0) and As (4.0).

Immunofluorescence and western blots reveal changes in protein expression of stage-specific markers of neuronal differentiation in response to CP treatment. While proliferating cells have low expression of β -tubulin III, differentiating cells have increased expression of β -tubulin III over time in non-treated cells. CP treatment results in overall dose-dependent decrease of cell viability but less change is observed with β -tubulin III expression (Figure 18). In western blot expression, while there is significant decrease of CP dose ($p = 0.022$), there is no significant difference between proliferation and differentiation hNPCs (Figure 20 A).

Other protein markers are altered by CP treatment in both proliferating and differentiating cells (Figure 20). Another neuronal marker, MAP2 shows dose-dependent increase of expression with CP treatment ($p = 0.044$). No significant difference between proliferation and differentiation hNPCs is shown. With arsenic treatment, more dramatic

increase of MAP2 expression is shown under differentiation conditions ($p=0.001$) while proliferation conditions did not change. This differential effect on proliferating and differentiating hNPCs were significant ($p=0.0001$) (Figure 20 B). Proliferation marker, PCNA, shows dose-dependent decrease of expression with CP treatment ($p=0.021$) but there is no significant difference between proliferation and differentiation hNPCs. Within proliferation cell culture conditions, dose-dependent decrease of PCNA expression is more dramatic especially at CP 20 $\mu\text{g}/\text{mL}$ ($p \leq 0.001$) (Figure 20 D).

Nestin does not have any significant dose-dependent changes with CP, but has a general increase in expression with CP 20 $\mu\text{g}/\text{mL}$ and As 1 μM treatment (Figure 20 C).

Caspase 3, an apoptosis marker expression has a general increasing dose-dependent trend with CP treatment; however these changes are statistically insignificant (Figure 20 E). pMAPK p42/44 (Erk1/2) is involved in many cellular pathways including cell proliferation, differentiation, and apoptosis. With CP treatment, pMAPK p42/p44 has a decreasing trend with CP treatment within proliferation conditions, but differentiating cells does not have noticeable changes with CP treatment while As treatment shows a significantly increased expression of pMAPK p42/p44 in differentiating conditions ($p=0.033$) (Figure 20 F).

In summary, β -tubulin III, MAP2, and PCNA has significant dose-dependent changes with CP treatment, though there is no differential effects between proliferation and differentiation.

Discussion and Future Direction

We established a human neural progenitor cell (hNPC) model to examine the early neurodevelopment effect to CP exposures during proliferation and differentiation. The cells were treated with either Proliferation Expansion Media (PEM) or HyClone Differentiation Media (HDM) with and without presence of CP (0-200 $\mu\text{g}/\text{mL}$). This wide range of CP concentration covers levels both below and above that those cause AChE inhibition. Because AChE is not

present in such early stages of differentiation and cell culture media are serum-free, there is no AChE activity or CP degradation due to binding to serum proteins in our culture. The CP concentration of interest was 0-20 µg/mL (0-57 µM) which is below the level of AChE inhibition. There is little literature quantifying the CP concentration or distribution in human brain after exposure. The concentrations used in this study were chosen based the available literature on CP and our system-based approach. In *in vitro* studies, CP concentration of 0-150 µM (0-52.5µg/mL) have been often tested for the neurotoxicity (Song et al. 1997; Das and Barone 1999; Caughlan et al. 2004; Guizzetti et al. 2005; Saulsbury et al. 2009)

The system-based approach is used to assess and predict developmental neurotoxicity across multiple levels of biological complexity. The effects of CP on *in vitro* hNPCs and cultured midbrain NPCs of mice are compared to the effects observed in an *in vivo* mouse study. The overall goal is to extrapolate findings to better understand the effects on human. Our *in vivo* study uses CP concentrations of 0-12 mg/kg/day which is below the level required for AChE inhibition. Human exposure to CP in residential area has decreased pesticide poisoning events since federal regulations have banned the residential use over concern for adverse effects to children (USEPA 2006; Blondell 2007). However, CP is still widely used in agriculture, thus exposure to CP as well as other organophosphate pesticides (OP) still remains for farmers, pesticide applicators, and their children. In a human exposure study done, farm worker's blood level for CP or other OP (e.g. Azinphos Methyl) levels were measured. The dose range (0.04-10 ng/g) includes concentration that inhibits AChE activity up to 20% in farm workers in blood. This is the level required for occupational action. Dosemetric conversion gives CP level in blood 10ng/g is equivalent to 10ng/mL. The lowest CP dose tested with *in vitro* hNPCs was 2.5 µg/mL and toxicity was evident at concentrations as low as 10 µg/mL. *In vitro* studies include higher concentrations of CP than are normally seen in human exposures or in animal studies. The reason is that *in vitro* experiments have exposure condensed into a short period of time while human or animals have subchronic exposure to low levels of CP for longer periods. CP and its

metabolites are eliminated from body rapidly. Health concerns remain, however, because farm workers are exposed to CP subchronically and, because CP is lipophilic, it can easily pass through cell membranes into the cytoplasm and deposit in cells as well as lipid tissues. Infant exposure levels have been measured from 0.08-0.16 mg/kg body weight in residential areas sprayed with CP to 0.7-2.1 mg/kg body weight (Alexander et al. 2006) and 2.1 µg/kg and 1.0 µg/kg for pesticide applicators and children, respectively, exposed to geometric mean of 11.3 - 30.9 µg/L of CP (Fenske and Elkner 1990; Alexander, Burns et al. 2006). Toddlers and fetuses in agricultural areas are a particularly vulnerable population, because they have fewer detoxifying enzymes and the blood brain barrier is not fully developed, allowing lipophilic chemicals such as CP to accumulate in the developing brain. Therefore, in order to observe the effects that may occur over subchronic exposures, testing for a higher concentration range is necessary. Effects observed at higher concentrations *in vitro* may inform potential effects *in vivo* following chronic exposures. The *in vitro* concentrations tested is high, it is still within the range of potential human exposure.

Exposure to Chlorpyrifos (CP) induced dose-dependent morphological changes and decreases in cell viability in both proliferation and differentiation culture conditions. Higher doses of CP (50-200 µg/mL) were included for the dose-response cell viability assays to validate that the doses of interest (0-20µg/mL) are reasonable to test on the proliferation and differentiation of hNPCs. Arsenic 1-4 µM was used as a positive control in both types of hNPCs. Although CP is a well-known acetylcholinesterase (AChE) inhibitor, there is also growing evidence for mechanisms of CP neurotoxicity that are independent of AChE inhibition (Slotkin, Seidler et al. 2007; Slotkin and Seidler 2009). Some of the mechanisms include oxidative stress, inhibition of neurite outgrowth and DNA damage (Eaton, Daroff et al. 2008; Howard et al. 2005; Oncu et al. 2002; Verma and Srivastava 2003; Slotkin and Seidler 2009; Giordano et al. 2007; Verma and Srivastava 2001). Epigenetic changes may also be an underlying mechanism of CP effects.

3 color staining showed differences in cell morphology between proliferation and differentiation hNPC conditions. In differentiating cells, CP treatment inhibited and altered neurite outgrowth. Differentiating cells treated with low CP concentrations (10 µg/mL) displayed less connected neurites whereas higher CP (50-100 µg/mL) had lost neurite connection entirely. Several *in vitro* studies of primary rat and mouse neurons confirm that they also have observed inhibition of neurite outgrowth (Axelrad et al. 2002; Howard, Bucelli et al. 2005). Apoptosis is characterized by its distinct morphological characteristics. In the developing brain, increased apoptosis after exposure to CP can cause structural changes that can lead to developmental alteration in brain function. With increased doses of CP, nuclear condensation, fragmentation, and blebs on cell membranes were evident (Figure 17). Cellular death was accompanied by apoptosis. This observation suggests that CP causes apoptosis as confirmed in many studies (Caughlan et al. 2004; Saulsbury et al. 2008; Saulsbury, Heyliger et al. 2009).

Oxidative stress induces a variety of cellular responses including apoptosis, and is known to be associated with neurodegenerative disease as well as developmental neurotoxicity (Beal 1995; Gitto et al. 2002). Caspase 3 expression, though insignificant, had a generally increasing trend with increased CP concentrations in both proliferation and differentiation hNPCs. CP is known to induce oxidative stress and cause cellular damages. Apoptosis via oxidative stress is caused by reactive oxygen species (ROS). While ROS is essential for normal differentiation and apoptosis, CP causes excessive ROS production which leads to oxidative stress, damages to DNA, proteins and membrane lipids, which results in cell death (Vollgraf et al. 1999; Martindale and Holbrook 2002; Giordano et al. 2007; Rafalski and Brunet 2011). Blebbing of cell membranes is associated with increased lipid peroxidation. The oxidative degradation of lipids causes damage to the cell membrane which consists mainly of lipids. Further assessment of oxidative stress will help understand the underlying pathways involved to this apoptosis.

CP can also induce apoptosis via Mitogen-Activated Protein Kinase (MAPK) signaling pathways such as c-Jun N-terminal Kinases (JNK), p38 MAPK, and Extracellular signal-Regulated Kinase (ERK 1/2) (pMAPK p42/44) in dopaminergic neurons (Caughlan, Newhouse et al. 2004; Ki et al. 2013). Our ENStem-A™ characterization so far have not identified the presence of dopaminergic neurons, thus initial assessment was to evaluate the changes in Erk1/2 (pMAPK p42/44) pathway. Our finding showed that pMAPK p42/p44 had significant differences between cell types with decreasing trend under proliferation cell culture conditions but no changes under differentiation cell culture conditions. Further investigation of JNK, ERK 1/2 and p38 MAPK activation would help understanding of whether apoptosis is induced by MAPK signaling pathways in proliferating and differentiating hNPCs.

CP induces loss of mitochondrial potential (Miguet-Alfonsi et al. 2002; Hemdan and Almazan 2006). Our finding from Alamar Blue cell viability assays suggests that 72hr after treatment differentiating cells are more sensitive than proliferating cells to CP concentrations above 20 µg/mL. However, during the early onset of differentiation (24 hrs after treatment), CP treatment affects differentiation cells less. Alamar Blue assesses the metabolic reductase activity of mitochondria of viable cells. Early apoptotic cells maintain mitochondria function, so even changes in apoptotic nuclear morphology are observed in early differentiation, cells still maintain mitochondrial function (Thompson 1995; Namgung and Xia 2000; Caughlan, Newhouse et al. 2004). 72 hrs after treatment, there is a significant decrease in mitochondrial potential induced by CP exposure in both proliferation and differentiation hNPCs. CP treatment induces decreased reductase activity of mitochondria at doses where nucleus condensation and fragmentation is observed. Our findings suggest that CP causes apoptosis with interference of mitochondrial function in both proliferation and early differentiation conditions and this change is more dramatic under differentiation culture conditions.

The changes of neuronal marker expression demonstrated a differential effect of CP on the neuronal cells during proliferation and differentiation. In proliferating cells, PCNA and β-

tubulin III expression was significantly decreased with increased CP concentrations. In differentiating cells, there was slight decreasing trend but this was insignificant. MAP2, another neural structural marker had increasing trend of expression with CP treatment in both proliferation and differentiation hNPCs. Differential alterations of neuronal protein markers during proliferation and differentiation by CP treatment may provide cell stage specific molecular and cellular mechanistic clues for understanding the potential effects of environmental agents on the neurodevelopment.

The differential effect of CP in proliferation and differentiation hNPCs is likely due to differences in regulation of ROS level, mitochondrial activity, and epigenetic changes. Regulation of ROS is important in balancing between self-renewal (proliferation) and differentiation of neural progenitor cells (Smith et al. 2000; Noble et al. 2005; Lekli et al. 2009; Rafalski and Brunet 2011). Cell proliferation maintains a better cellular response to mitogens in reduced intracellular environment and cellular responses of cellular differentiation and apoptosis increases in oxidizing environment (Noble, Mayer-Proschel et al. 2005). The presence of fibroblast growth factor (bFGF) in proliferation of NPCs reduces ROS level, suggesting that promotion of proliferation may act through regulation of ROS. This would help understand whether NPCs maintain their proliferation state or proceed to differentiation (Rafalski and Brunet 2011). A study using early postnatal mice reports that NSCs has higher rate of mitochondrial activity while having reduced ROS level than differentiated neuronal and glial cells (Madhavan et al. 2006). In addition, epigenetic regulation has been found to play a critical role during stem cell differentiation and fate specification (Sanosaka et al. 2009). Epigenetics usually refers to information on genetic regulation coded beyond the DNA sequence, such as DNA methylation or modifications to the chromatin structure by histone modifications. Genes can be either activated or silenced by epigenetic changes and these changes may be irreversible, resulting in adverse health outcomes later in life. Epigenetic factors appear to play a role in the neurodevelopmental effects associated with CP exposure (Moreira, Yu et al. 2010).

In summary, we have demonstrated that CP differentially effects on the proliferating and differentiating hNPCs. The mechanism of action includes apoptosis pathways independent of AChE inhibition and regulated by perturbation of mitochondrial function. Taken together, our findings demonstrate that CP can negatively affect the normal development of central nervous system.

Because children and fetus are frequently less able to detoxify toxic chemicals, they can be more vulnerable than adults (Bearer 1995; Mortensen et al. 1996; Lassiter et al. 1998). For example, in the case of CP, research for the University of Washington has shown that adult level activities of the detoxifying enzyme Paraoxonase 1 (PON1) are not achieved until later in child development (Chen et al. 2003; Furlong, Holland et al. 2006; Eskenazi et al. 2010; Huen et al. 2010). CP exposure during early development of brain can cause lifelong functional impairments (Rodier 1994). A coordinated review of the Children's Health Centers research has shown that CP exposure during pregnancy is associated with developmental delays and neurobehavioral defects, namely ADHD and decreased IQ (Eskenazi, Bradman et al. 1999; Young et al. 2005; Kofman et al. 2006; Rauh, Garfinkel et al. 2006; Bouchard et al. 2010; Eskenazi, Huen et al. 2010; Marks, Harley et al. 2010; Bouchard, Chevrier et al. 2011; Rauh, Perera et al. 2012; Saunders et al. 2012). Our *in vitro* studies demonstrate that CP exposure increases cell death via apoptosis and structural changes which can lead to developmental alteration in brain function (Marks, Harley et al. 2010).

Chapter 4: Suggestion for Future Direction

In this study, I established a model of human neural progenitor cell model of neuronal proliferation and differentiation, and characterized long-term dynamics of differentiating cultures. Furthermore, I applied this model to study the effects of CP on proliferation and differentiation hNPCs. Exposure to chlorpyrifos (CP) induced dose-dependent decreases of cell viability in both proliferation and differentiation culture conditions and had differential effects on the panel of protein markers tested.

hNPC cultures are challenging to work with because although basic composition of medium of differentiation is known (removal of growth factors FGF2 and EGF), the exact medium composition is unknown because of commercial protections. Also, the conditions required for specific neuronal differentiation is not fully characterized yet. For example, the plating density used in this research as 200,000 cells/mL is based on the commercial recommendation. However, the commercial supplier has recently modified to a higher cell density (500,000 cell/mL). Because density is a key driver of differentiation, we are also interested in testing higher cell density, micromass and 3D cultures of hNPCs to find whether this would give stronger signals for differentiation. Micromass may provide a better comparison with our previous *in vitro* mouse midbrain micromass culture. Ultimately, utilizing multiple human NPC lines with greater genetic diversity and gender difference would better represent the range of potential effects from chemical exposures.

Long-term differentiation of hNPCs showed neurite outgrowth, migration, and formation of networks and these changes got more extensive over time in culture. Expression of α -synuclein in later time points indicates that differentiating hNPCs in our culture system undergo functional maturation. Characterization of which brain region, what types of cells our hNPCs are differentiating to, and presence of neurotransmitter activity is of interest.

For both characterization of cells and cellular effects due to CP exposure, changes in neuronal marker expression using immunofluorescence and confocal imaging will allow characterization cell by cell expression. Confocal imaging will help illuminate the process of proliferation and differentiation in our culture.

CP induced neurotoxic effects on both proliferation and differentiation hNPCs. The mechanism of action includes apoptosis pathways independent of AChE inhibition and regulated by perturbation of mitochondrial function. Evaluating apoptotic cell death via oxidative stress, MAPK signaling pathway, and mitochondrial activity will help better understand the underlying mechanism. Also, as disrupted neurite outgrowth was observed, quantifying the damages of these neurite outgrowth would be our next step. Epigenetic factors are also thought to play a key role in stem cell differentiation and fate specification (Sanosaka, Namihira et al. 2009). Epigenetic factors regulate the fate of neural stem cells by turning genes on and off. Changes in epigenetic factors may also provide understanding of underlying mechanism of CP effects.

The established *in vitro* human neuronal proliferation cell model is an excellent model to capture the unique sensitivity of developing brain in these early stages of neuronal differentiation. The negative effects that CP and As on hNPC proliferation and differentiation provided insight that this hNPC model could be applied to other toxic compounds. It is a promising model for characterization of large number of drugs and environmental contaminants that remain untested for developmental neurotoxicity.

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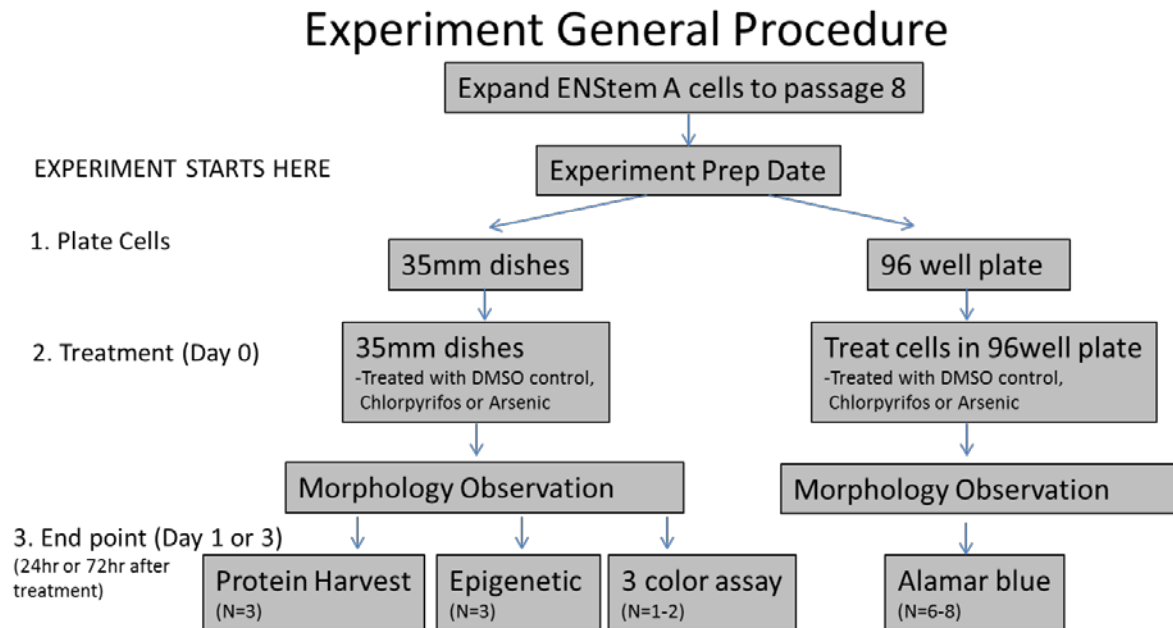
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Appendix

1. Overview of Experiment General Procedure.



Type of dish or plate and the total cell number seeded.

Type	Growth Area (cm ²)	Cell Concentration	Volume Added	Seeded Cell # (Cells)
35mm dish	9	200,000cells/mL	2mL	400,000
96 well plate	0.32	200,000cells/mL	100 µL	20,000

2. Experiment prep date and end point assays tested for CP study

	Prep_Date	12.09.09	12.10.09	1.11.10	1.19.10	2.08.10	3.01.10	3.22.10	6.21.10	10.01.10	11.04.10	1.30.11	12.29.11	1.25.12
Alamar Blue (Proliferation)	24hr	X	X	X	X									
	48hr	X			X									
	72hr		X	X	X									
	24hr	X	X	X	X									
Alamar Blue (Differentiation)	48hr	X			X									
	72hr		X	X	X									
3 color Assay (Proliferation)	24hr			X	X				X					
	48hr				X									
	72hr			X	X				X					
	24hr			X	X	X			X					
3 color Assay (Differentiation)	48hr				X									
	72hr			X	X	X			X					
Actin (Pro)	72hr				X		X	X				X	X	X
B_Tubulin III (Pro)	72hr						X	X				X	X	X
MAP2 (Pro)	72hr				X		X					X	X	X
Sox2 (Pro)	72hr				X		X	X				X	X	X
Caspase3 (Pro)	72hr						X	X					X	X
pMAPK_p42 (Pro)	72hr				X		X	X					X	X
pMAPK_p44 (Pro)	72hr				X		X	X					X	X
Nestin (Pro)	72hr				X		X	X					X	X
PCNA (Pro)	72hr				X		X	X					X	X
Actin (Dif)	72hr				X		X	X		X	X	X	X	X
B_Tubulin III (Dif)	72hr						X	X		X	X	X	X	X
MAP2 (Dif)	72hr				X		X	X		X	X		X	X
Sox2 (Dif)	72hr				X		X	X		X		X	X	
Caspase3 (Dif)	72hr						X	X					X	X
pMAPK_p42 (Dif)	72hr				X		X	X		X	X		X	X
pMAPK_p44 (Dif)	72hr				X		X	X		X	X		X	X
Nestin (Dif)	72hr				X		X	X		X			X	X
PCNA (Dif)	72hr				X		X	X		X	X		X	X

3. Protein marker test experiment prep date and Western blot run date information

Prep_date	Batch	Gel	Run	Dish	Actin (Pro)	Actin (Dif)	B-Tubulin (Pro)	B-Tubulin (Dif)	MAP2 (Pro)	MAP2 (Dif)	Sox2 (Pro)	Sox2 (Dif)	Caspase3 (Pro)	Caspase3 (Dif)	pMAPK P42 (Pro)	pMAPK P42 (Dif)	pMAPK P44 (Pro)	pMAPK P44 (Dif)	Nestin (Pro)	Nestin (Dif)	PCNA (Pro)	PCNA (Dif)	CCaspase3 17KDa (Pro)	CCaspase3 17KDa (Dif)	CCaspase 3.17KDa (Pro)	CCaspase 3.17KDa (Dif)	CCaspase 3.19KDa (Pro)	CCaspase 3.19KDa (Dif)
11910		Ge1.1	20510	a	x	x																						
11910		Ge1.1	22310	b	x	x			x	x	x	x			x	x	x	x	x	x	x	x						
30110		Ge1.1	32210	a	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x						
32210		Ge1.1	40410	a	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x						
100110		Ge1.1	111110	a	x	x																						
100110		Ge1.1	111110	b	x	x																						
100110		Ge1.1	111111	a		x																						
100110		Ge1.1	111111	b		x																						
100110		Ge1.1	111111	c		x																						
110410		Ge1.1	111111	a		x																						
110410		Ge1.1	111111	b		x																						
110410		Ge1.1	121610	a		x																						
110410		Ge1.1	121610	b		x																						
13011		GeL1	21211	a	x	x	x	x	x	x	x	x																
13011		GeL1	21211	b	x	x	x	x	x	x	x	x																
13011		GeL1	21211	c	x	x	x	x	x	x	x	x																
122911	A	GeL1	11112	a	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x						
122911	A	GeL1	11112	b	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x						
122911	A	GeL2	11112	c	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x						
122911	A	GeL2	11112	a	x	x																						
122911	A	GeL2	11112	b	x	x																						
122911	A	GeL2	11112	c	x	x																						
122911	B	GeL1	21112	a		x																						
122911	B	GeL1	21112	b		x																						
122911	B	GeL1	21112	c		x																						
122911	B	GeL2	21112	a		x																						
122911	B	GeL2	21112	b		x																						
122911	B	GeL2	21112	c		x																						
12512		GeL1	20812	a	x	x	x	x																				
12512		GeL1	20812	b	x	x	x	x																				
12512		GeL1	20812	c	x	x	x	x																				
12512		GeL2	20812	a	x	x																						
12512		GeL2	20812	b	x	x																						
12512		GeL2	20812	c	x	x																						

4. Epigenetic Histone Modification experiment prep date and Western blot run date information

Prep_date	Batch	Gel	Run	Dish	Histone 3 (Pro)	Histone 3 (Dif)	Acetyl Histone H3 (Lys9) (Pro)	Acetyl Histone H3 (Lys9) (Dif)	pAcetyl Histone H3 (Ser 10) (Pro)	pAcetyl Histone H3 (Ser 10) (Dif)	Acetyl Histone H3 (Lys18) (Pro)	Acetyl Histone H3 (Lys18) (Dif)	HDAC 4 (Pro)	HDAC 4 (Dif)	di-Methyl Histone H3 (Lys4) (Pro)	di-Methyl Histone H3 (Lys4) (Dif)	di-Methyl Histone H3 (Lys9) (Pro)	di-Methyl Histone H3 (Lys9) (Dif)	pdi-Methyl Histone H3 (Lys27) (Pro)	pdi-Methyl Histone H3 (Lys27) (Dif)	di-Methyl Histone H3 (Lys36) (Pro)	di-Methyl Histone H3 (Lys36) (Dif)	di-Methyl Histone H3 (Lys79) (Pro)	di-Methyl Histone H3 (Lys79) (Dif)	
62410		GEL1	71310	A	X	X	X								X	X									
62410		GEL2	71510	A	X	X	X								X	X									
62410		GEL1	72010	B	X	X	X								X	X									
62410		GEL2	71310	B	X	X	X								X	X									
62410		GEL1	71510	C	X	X	X								X	X									
62410		GEL2	72010	C	X	X	X								X	X									
62410		GEL1	11511	*pooled	X	X	X								X	X									
62410		GEL2	11511	*pooled	X	X	X								X	X									
62410		GEL3	11511	*pooled	X	X	X								X	X									
62810		GEL1	90910	A	X	X	X								X	X									
62810		GEL1	90910	B	X	X	X								X	X									
62810		GEL1	90910	C	X	X	X								X	X									
62810		GEL2	90910	A	X	X	X								X	X									
62810		GEL2	90910	B	X	X	X								X	X									
62810		GEL2	90910	C	X	X	X								X	X									
13011		GEL1	21211	A	X	X	X								X	X									
13011		GEL1	21211	B	X	X	X								X	X									
13011		GEL1	21211	C	X	X	X								X	X									
13011		GEL2	21211	A	X	X	X								X	X									
13011		GEL2	21211	B	X	X	X								X	X									
13011		GEL2	21211	C	X	X	X								X	X									
13011		GEL3	21211	A	X	X	X								X	X									
13011		GEL3	21211	B	X	X	X								X	X									
13011		GEL3	21211	C	X	X	X								X	X									
13011		GEL4	21211	A	X	X	X								X	X									
13011		GEL4	21211	B	X	X	X								X	X									
13011		GEL4	21211	C	X	X	X								X	X									
122911	A	GEL1	10712	A											X	X									
122911	A	GEL1	10712	B											X	X									
122911	A	GEL1	10712	C											X	X									
122911	A	GEL2	10712	A	X	X	X								X	X									
122911	A	GEL2	10712	B	X	X	X								X	X									
122911	A	GEL2	10712	C	X	X	X								X	X									
122911	A	GEL3	10712	A	X	X	X								X	X									
122911	A	GEL3	10712	B	X	X	X								X	X									
122911	A	GEL3	10712	C	X	X	X								X	X									
122911	A	GEL4	10712	A	X	X	X								X	X									
122911	A	GEL4	10712	B	X	X	X								X	X									
122911	A	GEL4	10712	C	X	X	X								X	X									

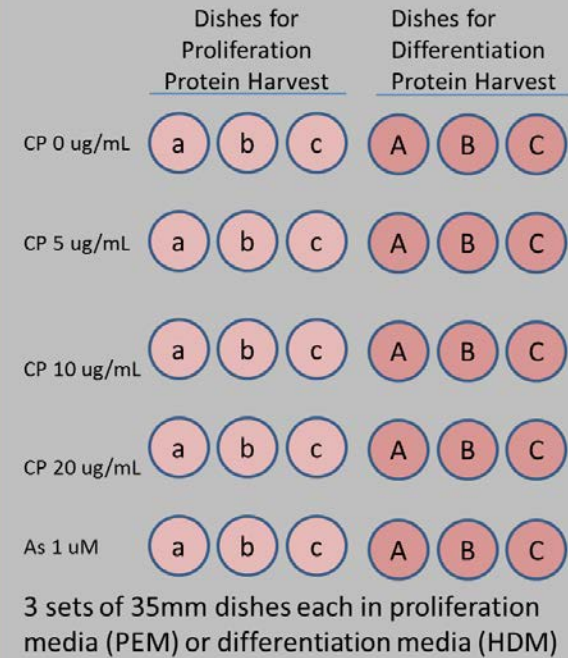
4. Epigenetic Histone Modification experiment prep date and Western blot run date information (continued)

Prep_date	Batch	Gel	Run	Dish	Histone 3 (Pro)	Histone 3 (Dif)	Acetyl Histone H3 (Lys9) (Pro)	Acetyl Histone H3 (Lys9) (Dif)	pAcetyl Histone H3 (Ser 10) (Pro)	pAcetyl Histone H3 (Ser 10) (Dif)	Acetyl Histone H3 (Lys18) (Pro)	Acetyl Histone H3 (Lys18) (Dif)	HDAC 4 (Pro)	HDAC 4 (Dif)	di-Methyl Histone H3 (Lys4) (Pro)	di-Methyl Histone H3 (Lys4) (Dif)	di-Methyl Histone H3 (Lys9) (Pro)	di-Methyl Histone H3 (Lys9) (Dif)	pdi-Methyl Histone H3 (Lys27) (Pro)	pdi-Methyl Histone H3 (Lys27) (Dif)	di-Methyl Histone H3 (Lys36) (Pro)	di-Methyl Histone H3 (Lys36) (Dif)	dj-Methyl Histone H3 (Lys79) (Pro)	dj-Methyl Histone H3 (Lys79) (Dif)		
122911	B	GEL.1	10612	A																						
122911	B	GEL.1	10612	B																						
122911	B	GEL.1	10612	C																						
122911	B	GEL.2	10612	A								X														
122911	B	GEL.2	10612	B								X														
122911	B	GEL.2	10612	C								X														
122911	B	GEL.3	10612	A																						
122911	B	GEL.3	10612	B																						
122911	B	GEL.3	10612	C																						
122911	B	GEL.4	10612	A																						
122911	B	GEL.4	10612	B																						
122911	B	GEL.4	10612	C																						
12512		GEL.1	13112	A						X																
12512		GEL.1	13112	B						X																
12512		GEL.1	13112	C						X																
12512		GEL.2	13112	A																						
12512		GEL.2	13112	B																						
12512		GEL.2	13112	C																						
12512		GEL.3	20112	A																						
12512		GEL.3	20112	B																						
12512		GEL.3	20112	C																						
12512		GEL.4	20112	A																						
12512		GEL.4	20112	B																						
12512		GEL.4	20112	C																						
12512		GEL.5	20412	A																						
12512		GEL.5	20412	B																						
12512		GEL.5	20412	C																						

5. Procedure of cell harvest and western blot

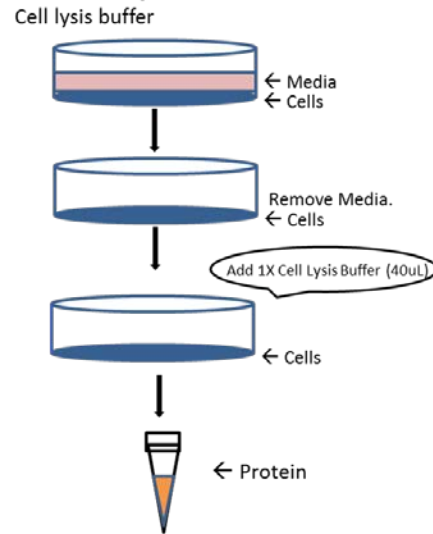
72hr Protein Harvest for Western Blot Procedure

1. Incubate hNPCs with & without treatment in PEM or HDM for 72hrs



2. At 72hr, harvest protein & store samples in -80°C freezer

Protein Expression Marker Tests

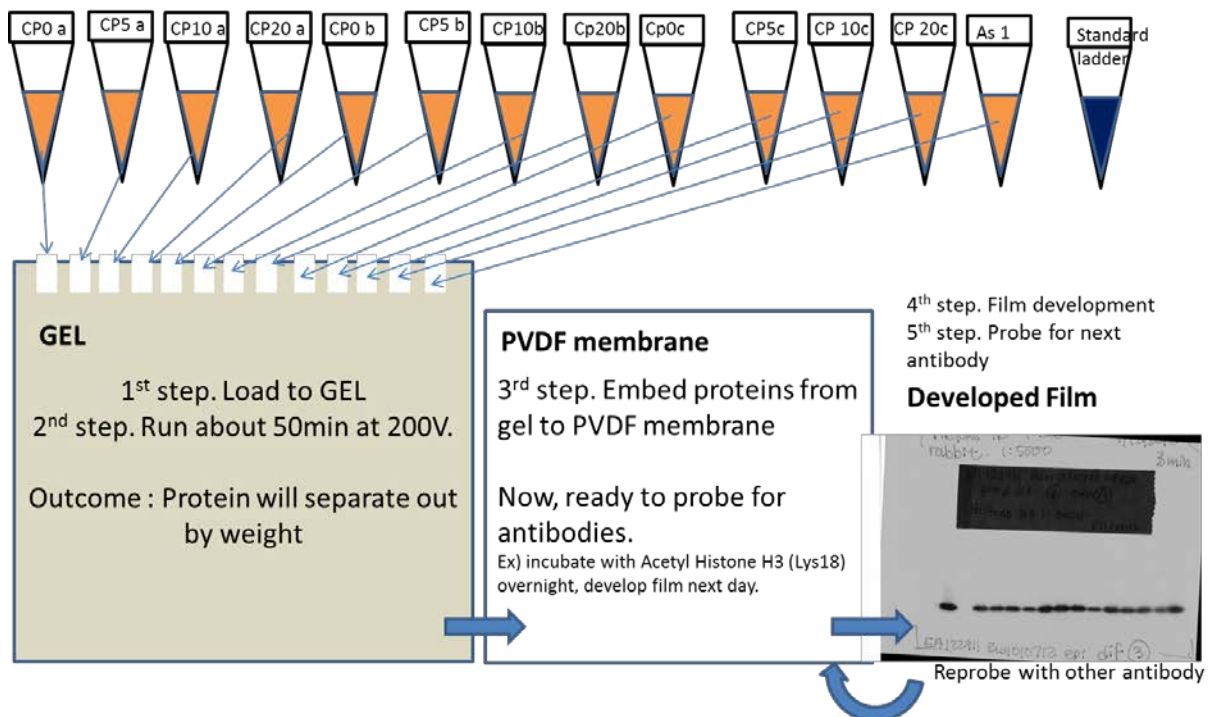


3. Protein Assay measures the level of protein in each sample

4. Make Western Sample(60 ug/80uL)

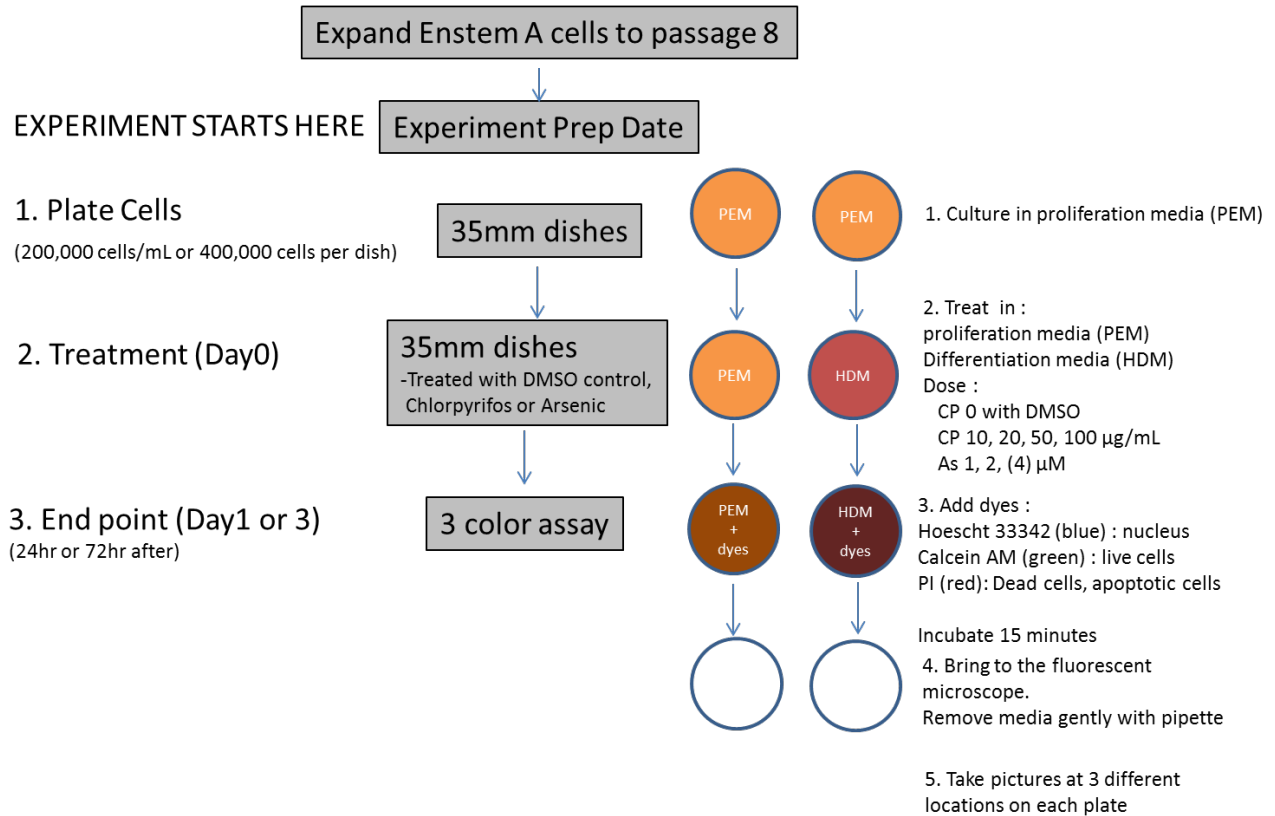
Western blot

15 well gel receives 15 uL sample per well ~11.25 ug protein per well



6.3 color assay procedure

3 color assay : General Experiment Procedure



3 color assay : Capture images at 3 different locations

