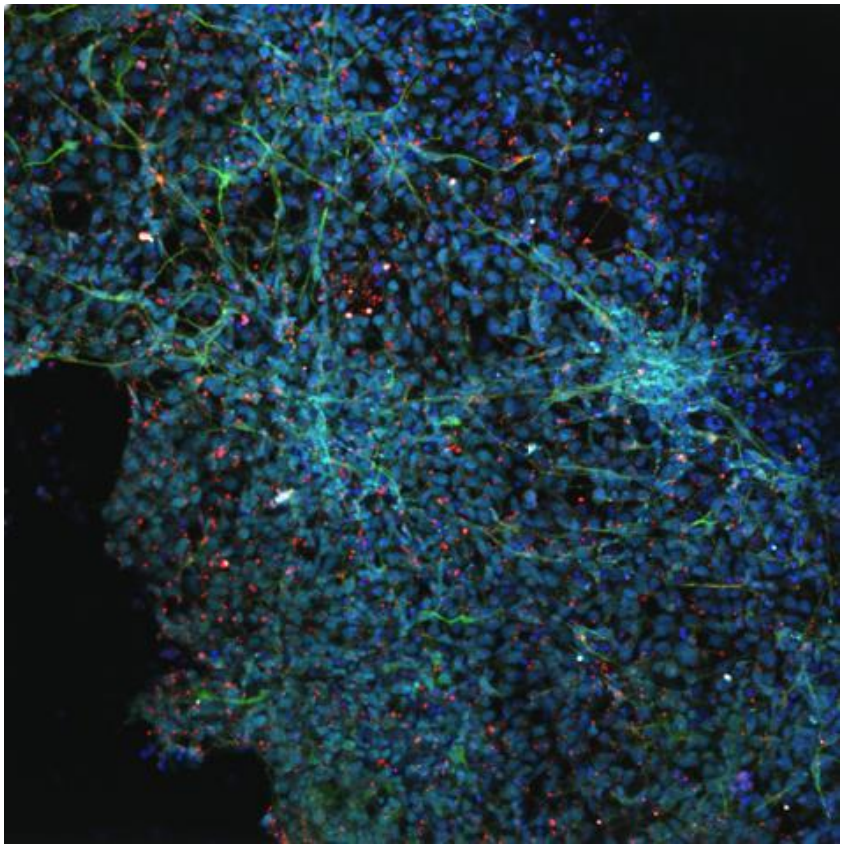
Master's Thesis

Master's Degree Program in Biomedicine

May 2021

Effects of the food processing contaminant acrylamide and its metabolite glycidamide in human neural stem cells undergoing differentiation



Malene Lislien

MABIO5900

60 ECTS

Faculty of Health Sciences

OSLO METROPOLITAN UNIVERSITY
STORBYUNIVERSITETET

Effects of the food processing contaminant acrylamide and its metabolite glycidamide in human neural stem cells undergoing differentiation

Malene Lislien

Norwegian Institute of Public Health

Department of Environmental Health

Section of Toxicology and Risk Assessment

Advisors: Oddvar Myhre, Jørn Andreas Holme, Ragnhild Paulsen and Anna Jacobsen Lauvås

Master Thesis submitted for Master's Degree in Biomedicine, 60 ECTS

Faculty of Health Sciences

Department of Life Sciences and Health

ACKNOWLEDGEMENTS

This thesis has been written as a part of the master's degree program in Biomedicine at Oslo Metropolitan University. All experimental work has been carried out at the Section of Toxicology and Risk Assessment, Department of Environmental Health at the Norwegian Institute of Public Health under the supervision of MSc Anna Jacobsen Lauvås. My main advisor was Dr. Oddvar Myhre at the Section of Toxicology and Risk Assessment, while Dr. Jørn Andreas Holme at the Section of Air Pollution and Noise and Professor Ragnhild Paulsen at the University of Oslo together with MSc Anna Jacobsen Lauvås were my co-advisors.

First, I would like to thank Anna for standing by me through what has felt like the toughest 10 months of my life. Thank you for all your patience, guidance, and enthusiasm when I finally got it right. Second, I would like to thank my co-student Inger Margit Alm for a great year and a wonderful collaboration. Having you by my side made the transition from thinking you know everything to realizing you know nothing much easier. Thank you both for making all those long hours in the lab a lot of fun, I will truly miss reading you the news and updating you on current events while you work.

I would like to extend my gratitude to Oddvar and Jørn for your guidance and valuable feedback during the experimental phase of the thesis as well as the writing process. Thank you, Tone Rasmussen, for always helping me find whatever I was looking for. I am grateful that you all took time out of your busy days to always answer my questions even though I had already asked them once or twice before and forgotten the answer. I would also like to thank Ragnhild for your insights during the work with this thesis.

Thank you to all the wonderful people I have met this year for making me feel so welcome. I would like to extent my gratitude to my friends and family for supporting me and keeping me sane in an otherwise different year. I would especially like to thank Helene and Guro for always believing in me.

Lastly, I would like to thank myself for not giving up.

Oslo - May 2021

Malene Lislien

ABSTRACT

Occupational exposure to the acrylamide (AA) monomer has been known since the 1960s to cause neurotoxicity of the central and peripheral nervous system. Concerns regarding exposure of the general populations arose upon discovering its natural formation in heated foodstuffs. AA is classified as a probable human carcinogen in addition to exhibiting genotoxic, reproductive, and developmental neurotoxic properties in experimental models. Adverse neurodevelopmental cognitive effects seen in animals have not yet been confirmed in humans. When absorbed from the gastrointestinal tract AA is metabolized mainly in the liver by CYP2E1 to glycidamide (GA). Both AA and GA are hydrophilic with wide distribution to organs and tissues. AA can reach the developing fetus via placental transfer and breast milk and prenatal exposure during pregnancy is associated with restricted fetal growth. It is unknown whether neurotoxicity is caused by AA itself or by GA. In this thesis, neural stem cells (NSCs) derived from human induced pluripotent stem cells were used to investigate the possible effect of exposure of AA and GA on key neurodevelopmental processes assessed by viability measurements, gene expression, and protein markers. The NSCs were differentiated and exposed to AA and GA ($1x10^{-8}$ – $3x10^{-3}$ M) for up to 21 days. Effects on cell viability were measured using Alamar Blue™ cell viability assay upon exposure for 1, 3, 14, and 21 days. Alterations in gene expression of five genes were assessed using real-time PCR after exposure for 3, 14, and 21 days. Protein expression was qualitatively examined using immunocytochemistry and high content imaging after exposure for 14 and 21 days. The NSC cultures differentiated as expected, judged by changes in morphology seen in phase-contrast microscopy, gene, and protein expression of unexposed cells. Exposure to AA and/or GA at $1x10^{-8}$ M resulted in increased viability, whereas millimolar concentrations induced cytotoxicity in a time- and concentration-dependent manner. No statistically significant alterations in gene expression were observed. However, some trends were found although these may be of limited biological relevance. Expression of the astrocyte marker *GFAP* was not detected and should be further investigated using immunocytochemistry. Protein expression of neurodevelopmental markers did not reveal large differences in fluorescence intensity upon exposure. In conclusion, exposure to human-relevant concentrations of AA and GA increased cell viability with the most apparent effects for immature neurons, whereas higher concentrations resulted in cytotoxicity. Statistically significant alterations in the selected gene or protein expression of markers related to neurodevelopment were not observed.

SAMMENDRAG

Siden 1960-tallet har det vært kjent at eksponering for akrylamid (AA) monomer forårsaker nevrotoksisitet i det sentrale og perifere nervesystemet. Bekymring for eksponering av den generelle befolkningen oppsto etter oppdagelsen av AA i varmebehandlet mat. AA er klassifisert som et mulig humant karsinogen i tillegg til å besitte gen-, reproduktivt- og utviklingstoksiske egenskaper i eksperimentelle studier. Alvorlige kognitive effekter sett i dyrestudier har ikke blitt bekreftet i humane studier til dags dato. AA tas opp fra mage-tarmkanalen og metaboliseres hovedsakelig i leveren av CYP2E1 til glysidamid (GA). Både AA og GA er vannløselige og distribueres i vannfasen til de fleste indre organer og vev. AA kan nå barnet gjennom overføring via morkaken og morsmelk og prenatal eksponering under svangerskapet er assosiert med begrenset fostervekst. Det er derimot ukjent om det er AA eller GA som forårsaker nevrotoksisiteten som er assosiert med eksponering. I denne oppgaven ble nevrale stamceller, opparbeidet fra humane induserte pluripotente stamceller, brukt til å undersøke effekten av eksponering på prosesser viktige i den humane hjerneutviklingen. Stamcellene ble differensiert og eksponert for AA og GA ($1x10^{-8}$ – $3x10^{-3}$ M) i opptil 21 dager og undersøkt ved bruk av viabilitetsmålinger, genuttrykk og proteinmarkører. AA og GA sin påvirkning på cellenes viabilitet ble undersøkt med Alamar Blue™ viabilitetsanalyse etter eksponering i 1, 3, 14 og 21 dager. Endringer i genuttrykket av fem utvalgte gener ble undersøkt ved sanntids-PCR etter eksponering i 3, 14 og 21 dager. Etter eksponering i 14 og 21 dager ble det kvalitative proteinuttrykket undersøkt med immuncytokjemi og høyoppløselighets-billedtagning. Kulturen utviklet seg som forventet vurdert etter endringer i morfologi ved bruk av fasekontrastmikroskopi, gen og protein markører av ueksponerte celler. AA og GA påvirket viabiliteten til cellene hvor $1x10^{-8}$ M medførte økt viabilitet mens millimolare konsentrasjoner resulterte i celledød på en tids- og konsentrasjonsavhengig måte. Det ble observert ikke-statistisk signifikante endringer og trender i genuttrykket, men som er av begrenset human relevans. Genuttrykket av astrocyttmarkøren *GFAP* ble ikke påvist og bør undersøkes nærmere med immuncytokjemi. Ingen større forskjeller i fluorescensintensitet ble observert for proteinmarkørene. I denne oppgaven ble det konkludert med at human relevante konsentrasjoner av AA og GA medførte en økning i viabilitet mest synlig for umodne nevroner mens høyere konsentrasjoner medførte celledød. Ingen statistisk signifikante endringer i gen- eller proteinuttrykk av relevante nevroutviklingsmarkører ble observert i denne studien.

ABBREVIATIONS

Acrylamide	AA
Adverse Outcome Pathway	AOP
Attention Deficit Hyperactivity Disorder	ADHD
Autism Spectrum Disorders	ASD
Bovine Serum Albumin	BSA
Brain-Derived Neurotrophic Factor	BDNF
cAMP response element-binding protein	CREB
Central Nervous System	CNS
Combination	50/50 mix of Acrylamide and Glycidamide
Days *in vitro*	DIV
Developmental neurotoxicity	DNT
Dulbecco‘s Modified Eagle Medium F-12 Nutrient Mixture	DMEM/F-12
Embryonic Day 42	E42
Ethylenediaminetetraacetic acid	EDTA
European Commission Joint Research Centre	EC-JRC
European Food Safety Authority	EFSA
Food and Agriculture Organization of the United Nations	FAO
Gamma Amino Butyric acid	GABA
Glial fibrillary acidic protein	GFAP
Glutathione	GSH
Glycidamide	GA
Growth-associated protein 43	GAP43
High Content Imaging	HCI

Hours	h
Human Embryonic Stem Cells	hESC
Human induced Pluripotent Stem Cells	hiPSC
International Agency for Research on Cancer	IARC
Joint FAO/WHO Expert Committee on Food Additives	JECFA
Limit of detection	LOD
Matrigel® Basement Membrane Matrix	BMM
Matrigel® Basement Membrane Matrix reduced growth factor	BMM rgf
Microelectrode assay	MEA
Microtubule-associated protein 2	MAP2
Neural cell adhesion molecule	NCAM
Neural Induction medium	NI medium
Neural Progenitor Cells	NPCs
Neural Stem Cells	NSCs
Neuronal Differentiation medium	ND medium
Non-Reverse Transcriptase	NRT
Norwegian Institute of Public Health	NIPH
Organization for Economic Co-operation and Development	OECD
Peripheral Nervous System	PNS
Phosphate Buffered Saline	PBS
Polymerase Chain Reaction	PCR
Post-Synaptic Density protein 95	PSD95
Relative Fluorescence units	RFU
Reverse Transcriptase	RT

Synaptophysin	SYP
The Norwegian Mother and Child Cohort Study	MoBa
Tyrosine Hydroxylase	TH
Vesicular Glutamate Transporter 1	VGlu1
World Health Organization	WHO

TABLE OF CONTENTS

1 INTRODUCTION

Acrylamide (AA) is a low molecular weight, water-soluble type-2 alkene[4]. It is an important industrial chemical primarily used in the production of polyacrylamide which is widely used in the paper, textile, and cosmetics industry as well as in construction, treatment of wastewater, and biochemical laboratory work[5, 6]. The AA monomer is neurotoxic, affecting both the central nervous system (CNS) and the peripheral nervous system (PNS)[4]. AA is classified as a probable human carcinogen by the International Agency for Research on Cancer (IARC) although this determination is based on experimental studies using research animals rather than human exposure through food[7]. Epidemiological studies suggest that dietary AA exposure is unlikely to contribute to increased cancer risk. Experimental studies mainly performed in research animals have found that AA is genotoxic and carcinogenic as well as exhibiting evidence of both reproductive and developmental toxicity[8].

Humans are exposed to AA orally, dermally, or by inhalation with the general population being predominantly exposed through gastrointestinal absorption, while industrial and laboratory workers may be exposed dermally and/or by inhalation[8]. When ingested AA is extensively absorbed through the gastrointestinal tract, distributed, and metabolized mainly in the liver with partial metabolism in peripheral organs[9]. AA is metabolized by epoxidation to glycidamide (GA) by cytochrome 450 enzyme CYP2E1 or by conjugation to glutathione (GSH)[5]. AA-GSH and GA-GSH-conjugates/adducts are further converted to mercapturic acid and excreted through the kidneys. Both AA-mercapturic acid/GA-mercapturic acid and AA-hemoglobin/GA-hemoglobin adducts are important biomarkers of exposure[8]. GA is highly reactive and hypothesized to be the cause of some of the toxic properties of AA. Both AA and GA are distributed into the total body water and thereby widely distributed into a wide range of organs including the brain and probably the fetus[8]. AA as a soft electrophile is more likely to form adducts with thiol- and seleno-groups on proteins and enzymes whereas GA, being a harder electrophile, also forms adducts with purines[9]. GAs ability to form DNA-adducts is believed to be the causality of the genotoxicity associated with AA[8].

1.1 Neurotoxicity of acrylamide in humans

The first report of AA toxicity in mammals came in 1958 followed by reports of AA poisoning of factory workers in the late 1960s[10]. Male industrial workers developed neurological symptoms after handling AA monomers when making flocculates for water treatment. The workers developed symptoms like muscle weakness, gait abnormalities, impaired reflexes, and

slurred speech. In most cases, the damage was reversible, and the workers regained full strength. The more severely affected workers got better after discontinuing work but remained ataxic[10]. The handling of AA monomer was suspended and the use of protective equipment when handling was recommended[10, 11].

Occupational exposure to AA occurred after handling of monomers during the production of polymerized products[10, 12] or by accidental exposure to the monomer[6, 13]. In the 1990s, in conjunction with tunnel construction, Norwegian and Swedish workers experienced symptoms coherent with AA poisoning[6, 13]. A chemical grouting agent, containing AA and N-methylolacrylamide, was used to stop extensive water leakage into the tunnels. As a result of the heavy water flow the polymerization process was not completed and the AA monomer was present in the leaking water. In Sweden, the leakage water was pumped into a nearby stream leading to AA poisoning of fish and cows in the surrounding area. After the construction work ceased, neurophysiological symptoms in all but one worker reversed[6]. In Norway, all workers experienced a full or partial reversal of symptoms upon discontinuing work[13].

1.2 Acrylamide in Food

The leakage of tunnel water into the environment caused a scandal in Sweden with food products from the region (risk zones) not allowed for sale due to concerns of possible contamination with AA. When assessing the possible degree of exposure to the populations living in the area a background level of AA-hemoglobin adducts was found in non-smokers without a known origin. The absence of AA-hemoglobin adducts in wild animals potentially exposed and the presence of AA in tobacco smoke indicated that heated food could be a source of the AA-hemoglobin adducts[14].

1.2.1 Formation of acrylamide in heated food

AA is mainly formed as a result of the Maillard reaction between asparagine and reducing sugars at temperatures exceeding 120 ºC[15]. The Maillard reaction generates both flavor and color in heat-treated food being responsible for the brown pigmentation forming on the surface[8]. The amount of AA formed differs depending on temperature, moisture, and the composition of amino acid and reducing sugars[8]. AA typically forms in plant-derived foods rich in carbohydrates and low in protein[16] during baking, frying, deep-frying, and overcooking of food. Formation accelerates towards the end of cooking when the moisture level falls and the surface temperature rises and will vary between different food types but also within the same type[16]. Svensson et al.[17] (2003) showed that among the foodstuffs analyzed potato products had the highest content of AA, possibly due to rich starch content. AA can also be found in coffee,

breakfast cereal, bread, crispbread, canned black olives, prunes, roasted nuts, and seeds as well as baby foods and infant formula[8].

1.2.2 Human exposure to acrylamide from food

In 2005 the Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that an intake of 1 µg/kg body weight (b.w) per day represented the average intake of the general population[16]. In an updated estimate from European Food Safety Authority[8] (2015) (EFSA) it was estimated that the average adult population (adolescents, adults, elderly, and very elderly) is exposed to 0.4-0.9 µg/kg b.w AA per day through diet. For the adult population, coffee is a significant contributor to AA intake representing 34% of the average exposure. Potato and fried products (except potato crisps and snacks) is the main contributor representing 49% of AA intake in the average adult population. Children are more exposed to AA through diet than adults with a daily exposure estimated at 0.5-1.9 µg/kg b.w. Children tend to prefer food rich in AA with the main contributor to AA intake being potato fried products (except potato crisps and snacks)[8]. After the assessment from EFSA in 2015 the Norwegian Scientific Committee for Food Safety did a risk assessment of the dietary exposure in the Norwegian population[18]. They found that in the adult, adolescent, and younger populations it was not likely that the Norwegian population had a different exposure to AA than other Europeans. However, Norwegian 1-year-olds had a higher consumption of infant porridge than the average European population representing 25% of the total energy intake in Norwegian toddlers[8]. In the Norwegian Mother and Child Cohort Study (MoBa) the main contributor to AA intake during pregnancy was snacks (which included potato chips, nuts, and popcorn), fried potatoes, and crispbread[19]. Because of AA's widespread exposure to the general population, Developmental neurotoxicity (DNT) is of concern.

1.2.3 Prenatal exposure to acrylamide

AA has been shown to cross the placenta both in *ex vivo* experiments as well as *in vivo* by detection of AA in umbilical cord blood. In an *ex vivo* placental perfusion study, both AA and GA were found to cross the placental barrier and reaching an equilibrium between the maternal and the fetal side after two hours (h). Because the placenta provides limited protection, the fetus may be likely to be exposed to AA and GA if present in maternal blood. As well as exhibiting high placental transfer, AA has also been detected in human breast milk. Fetal exposure through maternal diet can occur throughout gestation and continue after birth via breast milk.

A recent study gave a rough estimate of fetal plasma concentration of $2x10^{-9}$ M AA for mothers with an intake of 0.6-1.1 µg/kg b.w/day[9]. Pathways for possible fetal exposure is illustrated in Figure 1.

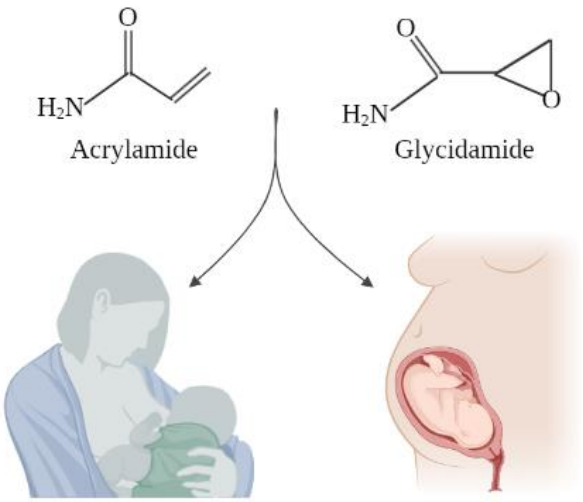


Figure 1. ***Molecular structure of AA and GA and illustration of possible pathways for fetal exposure.*** *AA and GA cross the placenta and may reach the fetus. AA has been detected in human breast milk. Figure created by Malene Lislien with biorender.com spring of 2021.*

1.3 Acrylamide in the environment

The occurrence of AA in the environment can originate from several sources, although the use of closed systems in the manufacturing of polyacrylamides makes this an unlikely source for environmental contamination. Environmental contamination may be due to leakage occurring during transport and storage as well as from the use and disposal of AA monomers or polymers[20]. Water leakage from the use of grouting agents can contaminate groundwater and soil[6, 8]. AA is enzymatically degraded by organisms and non-enzymatically by direct hydrolysis in water. AA concentration in the atmosphere is very low possibly due to AAs reactivity with hydroxyl radicals[8].

1.3.1 Human exposure to acrylamide from the environment

Non-dietary exposure to AA most commonly occurs via occupational exposure or through smoking. However, non-dietary exposure may also result from drinking water treated with polyacrylamide. The Council Directivity 98/83/EC has set a parametric value of AA monomer in drinking water to ensure the safety of lifelong consumption[8].

1.4 Human Neurodevelopment

Neurodevelopment and related processes are illustrated in Figure 2. At the very beginning of development, the embryo is a small oval-shaped, two-layered structure. These two layers contain two primitive cell types (epiblast and hypoblast) which in turn give rise to all the cells in the human body. During gastrulation, the epiblast cells will differentiate into three primary stem cell lines: the endodermal line, mesodermal line, and ectodermal line. The ectodermal line will again give rise to neural progenitor cells (NPCs) which further differentiate into all cell types occurring in the CNS[21].

The fetal period extends from the ninth week of gestation throughout the end of pregnancy and centers around neuron production, migration, and differentiation. From the end of gastrulation and through embryonic day 42 (E42) the NPC population expands rapidly by symmetric division. From E42 and to about halfway through gestation the progenitor cell population shifts from mainly proliferating to start differentiating. From this point, cell division shift from symmetric to asymmetric where every division produces one progenitor cell to maintain the stem cell population and one neuron. Neurons are post-mitotic meaning they lose the ability to divide and produce new cells. These neurons migrate from the proliferative zone to the developing neocortex. The first wave of migrating cells forms the preplate which, in turn, splits to the marginal zone and the subplate. Emerging between them is the cortical plate, the first cells to arrive here make the deepest layer of the cortex. Successively migrating cells will form the more superficial layers, in all six layers of neurons. Although both the marginal zone and the subplate largely disappear during the fetal period, the Cajal-Retzius cells in the marginal zone play an important role in cell migration and correct positioning of the different neurons into layers in the cortex. During development, the NPCs lose their ability to produce any neural cell line and become more restricted in the neurons they produce[21].

Once the immature neurons have reached their target region of the cortex they start developing into more mature neurons. To form information and communication networks with nearby neurons they develop dendrites and axons. Dendrites are specialized structures that receive input. Whereas each neuron has many dendrites in the immediate vicinity, neurons only have one axon. Axons are specialized structures for sending output and can extend long distances to form connections with other neurons. At the top of the axon, there is a structure called “a growth cone”, responsible for elongation and extension. Inputs from the microenvironment determines the direction of elongation and direct the axon toward its target[21]. Upon reaching its target a synapse is formed between the axon terminal of one neuron and the dendritic terminal of

another. The formation of new synapses is called synaptogenesis, an ability that neurons retain throughout their lifespan[22]. This type of synaptic plasticity forms the basis for learning and memory in the normal brain[23].

Neurons communicate through chemical messengers called neurotransmitters. Upon excitement, an action potential travels through the neuron as an electrical pulse and triggers the release of neurotransmitters from docked vesicles in the presynaptic neuron into the synaptic cleft. Neurotransmitters stimulate specific receptors on the postsynaptic neuron and influence this neuron's further actions. Based on the signal being excitatory or inhibitory the postsynaptic neuron will depolarize or neutralize. Most neurons secrete one of the small neurotransmitters, like gamma-aminobutyric acid (GABA) or glutamate, or the biogenic amine ligands, like dopamine, serotonin, epinephrine, and norepinephrine[24].

Regressive events during brain development involve programmed cell death and massive excessive production and pruning followed by systematic elimination resulting in loss of around 50% of the neurons in the region. Apoptosis takes place predominantly during the prenatal period while excessive production and pruning of connections largely take place postnatally. Uptake of neurotrophic factors protects against apoptosis and it's believed that developing neurons compete for such neurotrophic resources. This is called the neurotrophic hypothesis which implies that neurons must establish effective and functional connections in order to survive[21].

The developing brain exhibits an immature blood-brain barrier gradually developing *in utero* and is believed to be fully developed around 6 months after birth in humans. As a result, the developing brain may be more sensitive to perturbations of chemicals that would not affect the adult brain[22].

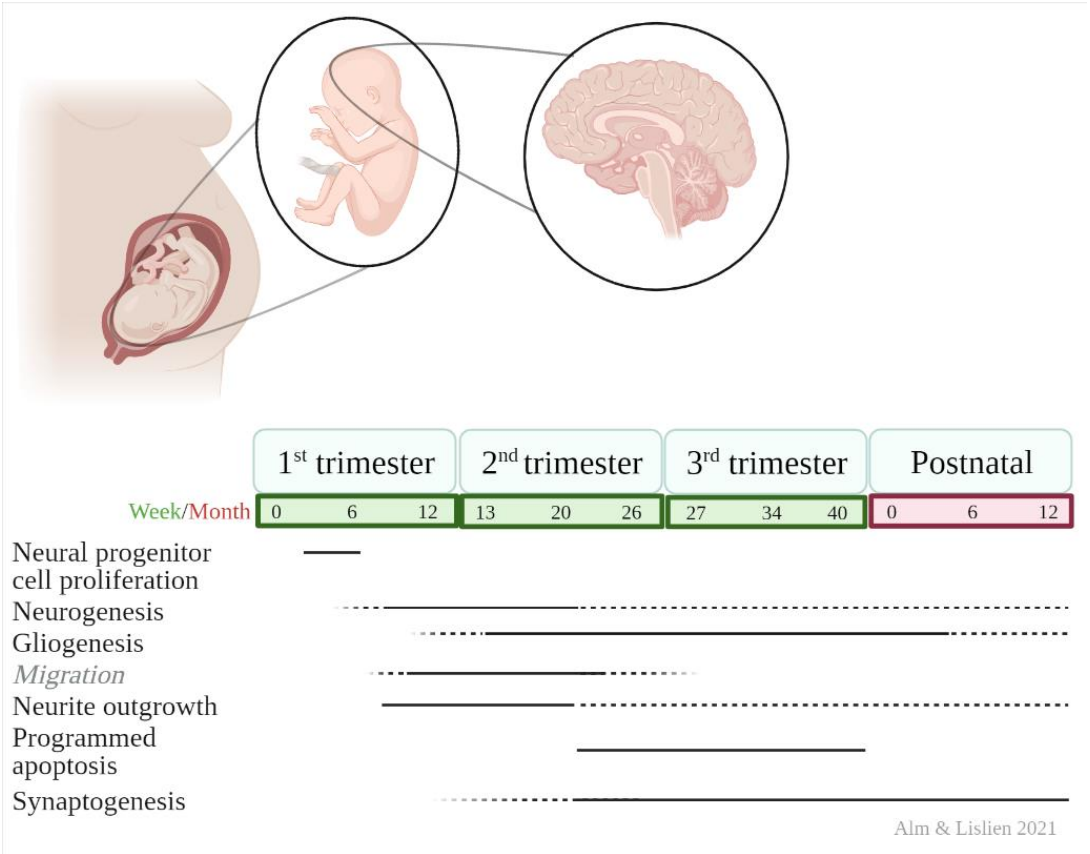


Figure 2. ***Overview of fundamental neurodevelopmental processes and developmental time windows relevant for DNT testing.*** *It is assumed that toxicants may cause DNT by interfering with at least one of these processes for normal brain development identified from in vivo studies. Developmental time windows are divided into "essential for neurodevelopment and vulnerable to toxic impact" (black line) and "time windows in which the process still occurs but is less sensitive for perturbations" (dotted line). Developmental processes measurable using 2D NSC models (in black font) and other processes essential for development measurable by 3D in vitro models (in grey italic). Figure created by Inger Margit Alm and Malene Lislien with biorender.com spring of 2021, inspired by [1] and [2].*

1.4.1 Effect of acrylamide on neurodevelopment

There are numerous studies of AA neurotoxicity in adult animals, however neurodevelopmental effects of prenatal and postnatal exposure are much less studied. Studies involving adult animals suggest that AA impairs adult neurogenesis (in the hippocampus) at low doses and adversely affects the cognitive function in male mice. DNT has mainly been investigated in rats showing morphological changes in the brain at the molecular and structural level[9]. Neurobehavioral alterations in rat offspring have also been reported after exposure to AA during gestation[9, 25].

DNT effects of AA have been investigated using *in vitro* cell models. It has been reported that AA attenuates differentiation processes in a few studies using neural stem cells (NSCs), with the most commonly reported endpoint being inhibition of neurite outgrowth[9]. Exposure to low concentrations of AA has been shown to increase cell number by sustaining proliferation and attenuating differentiation in cell-based *in vitro* models[26, 27], whereas high concentrations of AA affected proliferation and cell viability through oxidative stress and ROS generation[28, 29]. AA has also been found to affect neural migration, expression of neural cell adhesion molecule

(NCAM), which is important in synaptic plasticity, and other gene expression markers important in neurodevelopment. Long-term exposure to AA has been shown to affect neural differentiation processes more severely than short-term exposure. Low concentrations of AA alter the ratio between neural subpopulations in culture as well as the neuron/astrocyte ratio, without affecting viability[9].

Epidemiological studies of prenatal exposure to AA through maternal diet have shown associations between fetal growth restrictions and exposure to AA during pregnancy[9]. Prenatal exposure to AA through maternal diet was shown to negatively affect fetal growth, resulted in infants small for gestational age and low birth weight in the MoBa[19]. The NewGenesis European mother-child cohort study found a negative association between prenatal AA and GA exposure, measured by adduct formation in umbilical cord blood, and birth weight and head circumference[30]. Although AA's role in DNT is unknown[9] fetal growth restrictions are associated with adverse neurodevelopmental effects in children[31].

It is unknown what role GA plays in the neurotoxic properties associated with AA exposure. GA, being an epoxide and a hard electrophile, is more reactive with hard nucleophiles than AA[9], although its role in DNT is less studied. It has been hypothesized that because hard electrophiles tend to have genotoxic properties and softer electrophiles tend to cause non-carcinogenic toxicities like DNT, AA is the main contributor to DNT while GA causes the carcinogenic toxicities associated with AA exposure[9].

1.5 Neural Stem Cells derived from Human-Induced Pluripotent Stem Cells

Human induced Pluripotent Stem Cells (hiPSCs) are somatic cells reprogrammed by inducing overexpression of specific genes related to pluripotency. HiPSC can be induced to differentiate into a variety of human cells[32], including the NSCs used in this thesis.

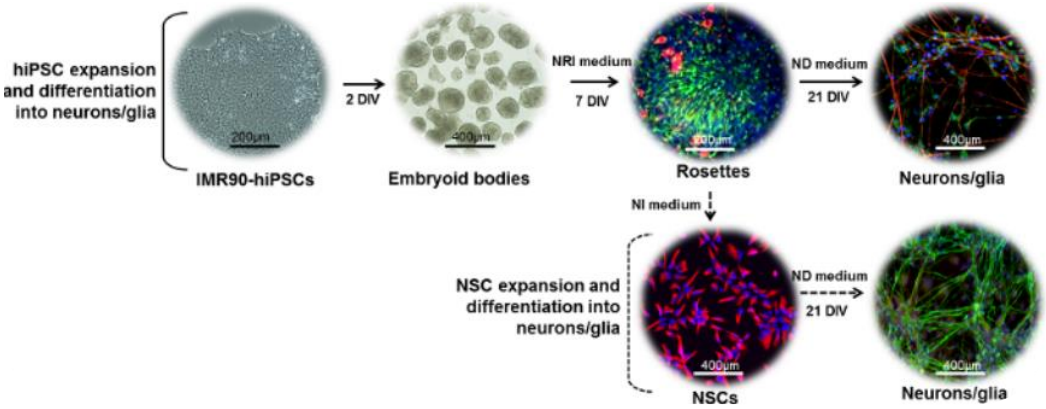


Figure 3. ***Schematic Illustration of the differentiation process from hiPSCs into NSCs used in this thesis.*** *IMR90-hiPSC colonies can be cut into fragments to form embryoid bodies (EBs). After 2 days* in vitro *(DIV), EBs can be plated onto laminin- or standard matrix-coated dished and cultured in the presence of neuroepithelial induction (NRI) medium to generate neuroectodermal derivates – rosettes. These rosettes can be dissociated, collected, replated on laminin- or standard matrix-coated dishes, and further differentiated into mature neuronal and glial cells in the presence of neuronal differentiation (ND) medium. NSCs derived from rosettes can also form neuronal and glial cultures in the presence of ND medium. Figure obtained with permission from the author, Francesca Pistollato[3],*

Previous characterization of the NSC model found a high percentage of the NSC marker nestin (60%) in undifferentiated cells as well as 7% of the cells in culture expressing Glial Fibrillary Acidic Protein (GFAP). nestin and GFAP are intermediate filaments abundant in NSCs and astrocytes, respectively[33, 34]. The expression of GFAP increases with the maturation of neural cells and is most abundant in the adult brain[33], while nestin is a marker for immature neurons and is expected to decrease upon differentiation and maturation[3]. The NSC model can be further differentiated into a more mature culture of neurons and astrocytes with an expected GFAP expression of 10-15% and 15-20% after 21 and 28 days of differentiation, respectively[3, 35]. Upon differentiating for 21 days and 28 days the culture is expected to retain a 20-30% expression of nestin as well as a decrease in the cell cycle marker Ki67, indicating neural progression into more mature subpopulations[3, 35].

Undifferentiated cells also express the neural marker β-III-tubulin (20%) and microtubule-associated protein 2 (MAP2) (18%), a cytoskeletal protein mainly expressed in maturing neurons[36]. Expression of *MAP2* is low in NPCs with increasing expression as cells differentiate[35, 36]. When neurons mature the location of MAP2 restricts to the cell body and dendrites[37], making MAP2 a marker for neurite outgrowth. Growth-associated protein 43 (GAP43) is a membrane-associated, neuron-specific phosphoprotein whose expression is associated with neural development and neurite outgrowth. *GAP43* was originally thought to represent a neuron-specific marker but it has since been discovered that it might also be expressed in non-neuron cells like glial cells. Expression of *GAP43* is thought to remain high during neural development[38] with a progressive increase along the course of differentiation[36].

In concordance with the decrease of nestin and Ki67, the NSC model differentiates into specific subpopulations of neurons. The NSC model being established at the Norwegian Institute of Public Health (NIPH) has been shown to consist of a large presence of glutamatergic (42%) and GABAergic neurons (11%) as well as a smaller percentage of dopaminergic neurons (20%) upon differentiation for 28 days[35]. The presence of these subpopulations of neurons suggests that the NSC model possesses forebrain and cortical-like features as well as midbrain specificity[3]. Glutamate is the main excitatory neurotransmitter in the CNS[39], whereas GABA is the most predominantly used inhibitory neurotransmitter[40]. Neurotransmission relies on the interconnections of neurons via synapses and the release of excitatory or inhibitory signals controlling the activity in the postsynaptic neuron[40]. The balance between excitatory and inhibitory signals is crucial for cortical function[41] and disturbances in this excitatory/inhibitory circuit are associated with neurodevelopmental disorders like Autism Spectrum Disorders (ASD) but also neurodegenerative disorders[39-41]. Dopaminergic neurons play a crucial role in motor behavior, motivation, and memory in the human brain where the loss of these neurons is closely associated with Parkinson's disease[42]. Dopamine dysregulation has been linked to schizophrenia[43].

Synapse formation and neural connectivity are important parts of neurodevelopment. The presynaptic and postsynaptic neurons connect and communicate through the release of neurotransmitters and are important for the survival and function of neurons constituting the CNS[32]. Expression of synaptic markers, neurite length and electrical activity have been shown to increase during differentiation in the NSC model indicating attempted synapse formation although these did not reach functional maturity[36]. Synaptophysin (SYP) and Post-Synaptic Density protein 95 (PSD95) can be used as *in vitro* markers for presynaptic and postsynaptic neurons, respectively[3, 44]. PSD95 is a scaffold protein abundant in excitatory synapses on glutamatergic neurons especially[44, 45], and it is important in neurodevelopment in terms of glutamatergic signal transmission, synaptic plasticity, and dendritic morphology[45]. In synaptic vesicles, SYP is the most abundant membrane protein and it is important in vesicle fusion as well as in the sustained release of neurotransmitters upon repeated excitement[46]. Synaptic dysfunction has been linked to neurological disorders like schizophrenia and ASD[45].

1.5.1 Ethical aspects of using *in vivo* vs *in vitro* test methods in developmental neurotoxicity testing

Russell and Burch's 3Rs, replacement, reduction, and refinement regarding the use of animals in research can be considered as the ethical norm in experimental research today[47]. The principle of the 3Rs is partially written into Norwegian law by *regulation concerning the use of animals for scientific purposes* which falls under the *Animal Welfare Act*[48]. In short, it states that the use of animals in research should be avoided if possible (replacement), as few animals as possible should be used to achieve the purpose of the experiments (reduction) and no unnecessary harm should incur animals participating in research (refinement). Replacement of animals used for research purposes has received much attention in recent years in the European countries. Establishment of the European Consensus-Platform for Alternatives (ECOPA) and the European Centre for the Validation of Alternative Methods at the European Commission Joint Research Centre (EC-JRC) in Italy coordinated efforts within cell-based toxicity testing in Europe[49]. The Norwegian equivalent to ECOPA, NorECOPA, is the consensus platform for alternatives to the use of research animals in Norway and promotes the use of the 3Rs in animal-based scientific research[50]. Development and use of human *in vitro* methods in substitute of animal models is a current paradigm shift in regulatory toxicology[51].

Assessment of DNT is not a mandatory requirement for routine regulatory toxicity testing in the European Union today and DNT testing is currently triggered only after observable modification in adult animals. The developing nervous system is more vulnerable to chemical exposures than the adult nervous system due to the complex, specific and time-sensitive aspects of brain development[1, 22, 52]. The complexity of the human brain and interspecies differences cause the use of animal models to not always identify DNT relevant for the human brain[1, 52]. The use of animals in research is also resource-intense regarding the number of animals needed, time, and overall cost of such experiments[52]. However, animal studies are used to investigate behavioral changes in addition to toxicokinetic studies following exposure to certain chemicals, an endpoint in which cannot be examined in cell-based models.

HiPSC-derived cultures are self-renewable, although not immortalized, avoiding tumor-growth-related genes to possibly affect the cell response to chemical exposure and do not exhibit age-related brain characteristics even after long-term culture. HiPSC-derived NSCs can be differentiated into mixed cultures of neurons and astrocytes which is particularly suitable for DNT testing. These models have also been found to express neurodevelopmental signaling pathways on both gene and protein level[35, 36]. Although the amount of glial cells is generally

low in *in vitro* models based on hiPSC it's considered suitable for studying chemically induced DNT[51]. Unlike human embryonic stem cells (hESC), hiPSC can be derived from consenting adults thus do not have the same regulations and ethical dilemmas as associated with hESC. HESC are isolated from blastocytes between day 5 and day 6 of development[53], a process which is highly regulated in many European countries including Norway[54], and raises ethical concern[3]. Work with hESC in Norway are regulated by "*bioteknologiloven – an act on human medical use of biotechnology*"[54] stating that hESC can only be used for research on severe illness in humans or to improve methods regarding *in vitro* fertilization whereas the use of commercially enquired hiPSC is not regulated by law[55].

1.5.2 Use of Adverse Outcome Pathways in research

DNT is an important endpoint in research and regulatory toxicology[56]. Epidemiological studies have shown correlations between exposure to certain chemicals and adverse effects on the developing brain[57]. These effects have mainly been observed on the population level or in animal studies. The increasing use of *in vitro* models poses an effective strategy to evaluate chemicals' potential for DNT effects[58]. Together with the use of an adverse outcome pathway (AOP), the NSC model can be used to create a better mechanistic understanding of key events on a cellular level mediating adverse effects on the individual level[51, 58]. An AOP is a linear simplification of a series of measurable key events originating from one molecular initiation event causing cellular, structural, or functional alterations observable at the individual or population level as an adverse effect. It is based on the belief that toxicity originates from a chemical interaction with a biomolecule in the body, which induces a perturbation in a biological process and thereby impairs a function critical for normal function and/or development. This impaired function is observed as an adverse outcome on the individual level or population level[59]. The NSC model differentiated into a 2D culture of neurons and astrocytes recreates key events important in human brain development including proliferation, differentiation, synaptogenesis, network formation, cell death/injury and function[57]. In DNT, the observable adverse outcome typically involves cognitive damage, impairment in learning and memory, lowered IQ, and behavioral deficits and involve disorders like ASD, schizophrenia, and Attention Deficit Hyperactivity Disorder (ADHD)[57, 60]. By connecting single AOPs, a more realistic representation of disease pathophysiology emerges with multiple molecular initiating events leading to a single adverse outcome and vice versa[60]. The use of *in vitro* methods to describe causative relationships between molecular initiation events, key

events, and adverse outcomes is an important tool in regulatory toxicology[61] and can be used to determine causation of DNT rather than correlations.

2 AIM OF STUDY

This master thesis is a part of a larger project to establish the NSC model at the NIPH. The NSC model is a human *in vitro* alternative to animal research that captures processes relevant to human brain development. The model's development and establishment at NIPH is part of a current paradigm shift in regulatory toxicology towards the use of non-animal approaches and can be differentiated into mixed cultures of neurons and astrocytes.

By taking part in the overall establishment of the NSC model at NIPH, I participated in experiments with the aim to characterize and clarify the function of the model in the laboratory.

I. Characterizing morphological changes in the cultures as it develops by phase-contrast microscopy
II. Optimizing assay for cell viability measurements
III. Establishing assay for analysis of gene expression related to neurodevelopment
IV. Establishing assay for immunocytochemical analysis of protein/enzyme markers related to neurodevelopment using high content imaging techniques

Epidemiological studies suggest that exposure to AA and/or its metabolite GA during pregnancy has an impact on fetal growth, however, to our knowledge, no human studies investigating cognitive function in children upon exposure through maternal diet have been published to this date. Experimental studies (*in vivo* and *in vitro*) indicate that exposure to AA may cause neurodevelopmental toxicity. The mechanisms involved, as well as the relative potency of AA and GA, are largely unknown. Our overall hypothesis is that AA and/or its metabolite GA cause changes in neurodevelopmental processes at the cellular level recreated in our human NSC model. If so, this may provide information regarding relative potency and possible mechanisms involved. More specifically we hypothesize that exposure to AA, GA, and/or their combination cause alterations in:

I. Cell viability
II. Gene expression of markers related to neurodevelopment
III. Protein markers related to neurodevelopment and morphology

Markers related to neurodevelopment included in this thesis represent processes like proliferation, differentiation, synaptogenesis, and neurite outgrowth.

3 MATERIALS AND METHODS

The experiments in this master thesis were performed at NIPH, as a part of a larger project aiming to establish the NSC model at NIPH and assess transferability of the model from EC-JRC, where protocols for culturing of the cells were originally developed. Part of the experiments performed was validated using information from EC-JRC as a reference point.

3.1 Chemicals

For specific information regarding chemicals used in this thesis, see appendix 2.

3.2 Neural Stem Cells

Specific information regarding the maintenance of cells, cryopreservation, culture medium, and coating of labware is described in the protocols in appendix 1.

For this experiment, NSCs derived from the hiPSC-line IMR90 were used. IMR90 is a fibroblast line originating from the lung tissue of a human female[62], induced by viral transfection of *Oct4* and *SOX2* transcription factors (using pMIG vectors) to hiPSC. IMR90-hiPSC was further differentiated into the NSCs we use in this thesis as detailed in[3]. NSCs must be passaged 4-5 times before inducing differentiation[3].

The NSCs (kindly provided by EU-JRC) were cultured in Neural Induction (NI) medium (A1647801, Thermo Fisher, USA) at 37 ºC in a 5% CO_2 humidified atmosphere. All work with cells was done using standard aseptic techniques. NSCs were cryopreserved in CryoStor Cell cryopreservation medium (C2874-100ML, Sigma-Aldrich, USA) containing 10% DMSO and kept at -150 ºC. NSCs were passaged at 95-100% confluency (once a week) by enzymatic dissociation using 0.05% Trypsin-Ethylenediaminetetraacetic acid (EDTA) (15400054, Thermo Fisher, USA) and replated on Basement Membrane Matrix (BMM) Matrigel (354234, Corning, USA) coated flasks (353108/353136/355001, Flacon, USA) in NI Medium, changed three times a week for cells kept in expansion. NSCs were differentiated on BMM reduced growth factor (354230, Corning, USA) coated plates for up to 21 days into mixed cultures of neurons and astrocytes in presence of Neural Differentiation (ND) Medium (21103049, Thermo Fisher, USA), refreshing medium twice per week.

3.2.1 Passaging of cells

The cell medium was aspirated, and the cells were incubated in 0.05% Trypsin-EDTA at 37 ºC for 2 min. Trypsin was inactivated by mixing 1:2 with Defined Trypsin Inhibitor (R007100, Thermo Fisher, USA) at 37 ºC. The cell suspension was then centrifuged at 130 g for 4 min and 30 sec and the supernatant was aspirated carefully making sure to avoid aspirating the cell

pellet. The cells were then gently resuspended in NI medium and counted on an automatic cell counter (LUNA II, Logos Biosystems, South Korea) using a 1:2 mix of cell suspension and Trypan Blue (T8154-100ML, Sigma-Aldrich, USA). After passaging cells were plated as described for every experiment and seeded in flasks for future passages as described in 3.2.

3.3 Alamar Blue™ cell viability assay

Cell viability after exposure to AA and GA on NSCs undergoing differentiation was assessed using Alamar Blue™ cell viability assay. Alamar Blue™ Cell Viability Reagent (DAL1025, Invitrogen, USA) contains resazurin, which is a non-toxic, water-soluble compound with a blue, non-fluorescent color. Resazurin acts as an intermediate electron acceptor in the electron transport chain without interfering with normal metabolic activity in the cell, allowing for continuous monitoring of cells in culture. When resazurin enters living cells, it will be reduced by mitochondrial dehydrogenase to resorufin, which is pink and highly fluorescent. Depending on time, resorufin will achieve equilibrium with dihydroresorufin which is colorless leading to a decrease in fluorescence. The development of fluorescence is proportional to the number of living cells; dead or damaged cells will reduce resazurin at a lower rate than living cells and will give a lower fluorescent signal. As modifications of cell proliferation and/or metabolic activity may influence the fluorescence, this assay will not necessarily directly reflect cell death. Alamar Blue™ assay was chosen for assessment of cell viability as it is non-toxic, and thus allows for further incubation of cells so that optimal incubation time for measuring toxicity can be found. A protocol for the Alamar Blue™ assay has been previously established by EC-JRC for assessment of cell viability of the NSC culture[63].

3.3.1 Optimization

The protocol for Alamar Blue™ cell viability assay was optimized by examining the effect of cell density, incubation time, and substrate concentration on viability measurements. The purpose was to find the optimal incubation time and substrate concentration for future experiments as well as assessing a possible impact of cell densities on cell viability. A known cytotoxic concentration ($1x10^{-3}$ M) of GA was used as a positive control for 80-90% cell death (unpublished pilot study). Two substrate concentrations were chosen to compare commercial protocols from Invitrogen[64], used by NIPH and Promega[65], which is used at the EC-JRC. The optimization was performed by trained personnel at NIPH while the student projects, performed in parallel, was mainly aimed at cell cultivation training and further validation of the results. Although two identical student projects were performed, the data was collected individually.

3.3.1.1 Experimental design

To investigate the effects of cell density on the Alamar Blue™ assay, 7000 cells/well were plated in 96-well plates (354461, Corning, USA). Cells were then allowed to proliferate for either 1 or 2 days in NI medium before differentiated and exposed in ND medium for 1 and 3, or 1 and 5 days, respectively. Human cells proliferating in culture typically divide every 24 h whereas upon inducing differentiation neurons withdraw from the cell cycle and enter a terminally differentiated G_0 state where cell division rarely occurs[32]. The purpose was to determine the optimal proliferation time suitable for the detection of changes in cell viability after 1-5 days of differentiation. The optimization performed by the NIPH included additional cell densities, 10500 cells/well and 14000 cells/well, for a more controlled assessment of the cell densities possible impact on the analysis. The experimental design is shown in Figure 4.

Alamar Blue™ Cell Viability Reagent was added to the cell medium in a final concentration of either 1:10 (Invitrogen[64]) or 1:6 (Promega[65]) (see Table 1) upon differentiating for either 1, 3, or 5 days in ND medium with or without $1x10^3$ M GA. A blank consisting of ND medium only and a negative control consisting of cell medium and Alamar Blue™ in final concentrations of 1:10 and 1:6 was included. The cells were then incubated at 37 ºC for 8 h and fluorescence was read once every h from the bottom up with 520-540 nm/580-600 nm (excitation/emission) with CLARIOstar plate reader (BMG LABTECH, Germany) and a gain of 850.

Table 1. ***Added volumes of medium and Alamar Blue™ Cell Viability Reagent during optimization and cytotoxicity experiments****. Amount of Alamar Blue™ added to wells was calculated from the dilution used based on the preexisting ND medium in wells. Blank included total volume in wells for both dilutions.*

	Optimization		Cytotoxicity
Alamar Blue™ Dilution	1:6	1:10	1:10
Volume of cells + ND medium	135 µl	135 µl	135 µl
Added Alamar Blue™	27 µl	15 µl	15 µl
Volume Negative Control (ND medium + Alamar Blue™)	135 µl + 27 µl	135 µl + 15 µl	135 µl + 15 µl
Volume of Blank (ND)	162 µl	150 µl	-
Total volume in well	162 µl	150 µl	150 µl

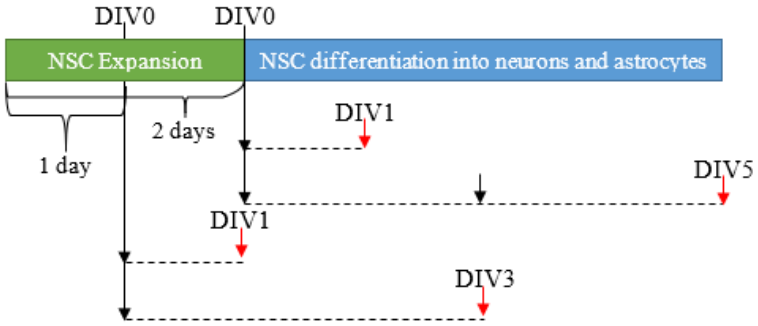


Figure 4. ***Experiment design for optimization of Alamar Blue™ for NSC****. Black arrows represent medium change with exposure to AA and/or GA. Red arrows represent stop of differentiation and start of Alamar Blue™ analysis. The student project was done in parallel to the validation performed by trained personnel at NIPH with the same experimental design, only differing in cell densities assessed. DIV = days of differentiation and exposure.*

3.3.2 Cell viability experiment

Alamar Blue™ assay was performed to assess possible cytotoxicity in NSCs undergoing differentiation by measuring the metabolic activity after exposure to different concentrations of AA and GA. Determination of possible cytotoxicity due to exposure to AA and GA was important for further analysis of potential alterations in gene expression markers and/or protein markers relevant to neurodevelopment.

3.3.2.1 Preparation of test substances

3 M master stock solutions were prepared by dissolving AA and GA in sterile water by a trained professional at NIPH. A combination was made by mixing AA and GA 1:2, ensuring a total concentration similar to the AA and GA master stocks. Stock dilutions were prepared as described in appendix 3 with concentrations of 3 M, 1 M, $3x10^{-1}$ M, $1x10^{-1}$ M, $3x10^{-2}$ M, $1x10^{-2}$ M, $3x10^{-3}$ M, $1x10^{-3}$, $3x10^{-4}$ M, $1x10^{-4}$ M, $3x10^{-5}$ M, and $1x10^{-5}$ M. Preparation of stock dilutions was done once to minimize exposure variations and frozen at -80 ºC between use. See table in appendix 3 for detailed information.

3.3.2.2 Experimental design

Concentration-dependent cytotoxicity of AA and GA was investigated after 1, 3, 14, and 21 days of continuous exposure and differentiation, experiment design is illustrated in Figure 5. Stock dilutions were added to the medium in a 1:1000 dilution, resulting in final concentrations of: $3x10^{-3}$ M, $1x10^{-3}$ M, $3x10^{-4}$ M, $1x10^{-4}$ M, $3x10^{-5}$ M, $1x10^{-5}$ M, $3x10^{-6}$ M, $1x10^{-6}$ M, $3x10^{-7}$ M, $1x10^{-7}$ M, $3x10^{-8}$ M and $1x10^{-8}$ M and a water content of 0,1 %. The NSCs were seeded at the recommended cell density of 7000 cells/well in 96-well plates in NI medium. After 1 or 4 days of expansion, cells were exposed to AA, GA, or a 1:2 mixture of the two prepared fresh in ND medium. Cell medium with exposure was refreshed twice a week.

Alamar Blue™ assay was performed after 1, 3, 14, and 21 days of continuous differentiation and exposure to test substances. Analysis was performed as described in 3.3.1.1 with 1:10 dilution and 3 h and 30 min incubation time before analysis by CLARIOstar. The plate layout is shown in appendix 4 and the volumes added are shown in Table 1. The results were corrected to the negative control, to correct for background fluorescence caused by reagent in medium[66], and normalized by the mean of solvent control. Residual Alamar Blue™ was aspirated and plates were kept in -80 ºC freezer pending RNA isolation.

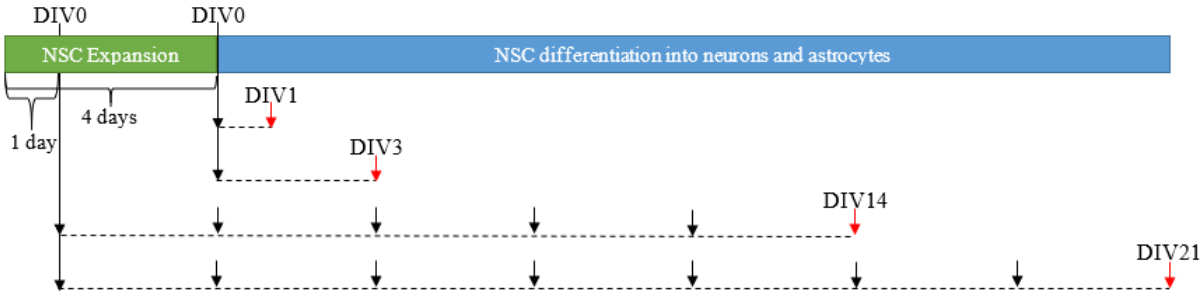


Figure 5. ***Experiment design for cytotoxicity experiment****. Black arrows represent medium change with exposure to AA and/or GA. Red arrow represents stop of differentiation and exposure, start of Alamar Blue™ analysis and storage for further RNA isolation. DIV = days of differentiation and exposure.*

3.4 Gene expression analysis using real-time polymerase chain reaction

Real-time Polymerase Chain Reaction (PCR) is a common tool in toxicology used for the quantification of gene expression[67]. TaqMan real-time PCR uses fluorescence for the detection of a target in real-time. A TaqMan probe contains a reporter dye, in this experiment FAM, and a quencher that represses the fluorescence emitted by the dye when in immediate proximity. The TaqMan probe anneals to the target of interest while the primers anneal just outside of the target of interest. During the extension phase of the real-time PCR the Taq polymerase, a thermostable polymerase with 5‘-exonuclease activity cleaves the probe causing dissociation of the report dye from the quencher. This results in a fluorescence signal proportional to the amount of PCR product in the sample[67].

3.4.1 Preparation and exposure to test substances

For the determination of possible effects of exposure to AA and GA on gene expression markers related to neurodevelopment real-time PCR analyses were performed. Three differentiation and exposure timepoints, 3, 14, and 21 days, were further investigated for alterations in gene expression after exposure to three non-cytotoxic concentrations of the chemicals: $1x10^{-5}$ M, $1x10^{-6}$ M, and $1x10^{-7}$ M. Sample preparation were performed as described in 3.3.2.2.

3.4.2 RNA purification

Total RNA was isolated using the RNeasy Mini kit (74106, Qiagen, Germany) according to suppliers' protocol[68] with minor modifications. The cells were lysed by adding 75 µl of RLT lysis buffer directly to the wells, following incubation at room temperature for 5 min. The lysate was transferred to a QiaShredder column and centrifuged for 5 min at 12.000 g. Depending on the number of replicate wells in the plate, the lysate was centrifuged once or twice to not exceed the maximum volume of the QiaShredder column. The lysate was stored at -80 ºC awaiting RNA isolation.

Before isolation, the lysate was thawed and mixed with one volume of 70% ethanol. The lysate mixed with ethanol was transferred to an RNeasy Spin column and centrifuged twice or thrice for 0.5-1 min to not exceed the maximum volume of the Spin column. The flow-through left in the collection tube after a washing step was always discarded, leaving total RNA bound to the membrane in the Spin column. The RNA was then washed with RW1 buffer and treated with DNase I for 15 min at room temperature to remove all genomic DNA. The filter was then washed once with RW1 and twice with RPE buffer before the filter was dried by centrifugation for 1 min. Total RNA was eluted in 50 µl of RNase and DNase free H_2O added directly to the center of the Spin column and measured using spectrophotometry on Nanodrop 1000 (Thermo Fisher, USA).

3.4.3 cDNA synthesis

cDNA synthesis was performed using High Capacity cDNA Reverse Transcription kit from Thermo Fisher (4368814, Thermo Fisher, USA). Reverse transcription was performed using 1000 ng total RNA and a non-reverse transcriptase control (NRT) was included. Master Mix, including Master Mix for NRT, was prepared as described by the manufacturer, see Table 2 for preparation details, and mixed with diluted RNA. cDNA synthesis was run using a CFX96™ Real-Time System (Bio-Rad, Germany) with a 10 min warm-up at 25 ºC for primer annealing, synthesis for 2 h in 37 ºC and a 5 min enzyme deactivation at 85 ºC. After synthesis, complete cDNA was stored in -20 ºC pending real-time PCR analysis.

Table 2. ***Preparation of Master Mix for gene expression analysis using real-time PCR.***

Master Mix Reagent	Volume per sample (µl)	Non-reverse transcriptase control (µl)
10x reverse buffer	2.5	2.5
25x dNTP	1	1
10x Random primers	2.5	2.5
Multiscribe reverse transcriptase	1.25	-
Nuclease free water	5.25	6.5
Diluted RNA (80 ng/µl)	12.5	12.5
Total volume	25	25

3.4.4 Real-time polymerase chain reaction

Master Mix consisting of TaqMan Gene Expression Master Mix (4369016, Applied biosystems, USA) and TaqMan gene expression assays (4331182, Applied biosystems, USA) was prepared fresh before every run and mixed with prediluted cDNA samples, resulting in a final cDNA dilution of 1:50. Real-time PCR efficiency and optimal cDNA concentration were previously determined by running dilutions series on pooled cDNA by trained personnel at NIPH and cDNA were diluted to a final concentration of 1:50. All samples were run in triplicate in a 384-well plate (HSP3805, Bio-Rad, Germany), 10 µl of each sample, and run on CFX 384™ Real-Time Systems (Bio-Rad, Germany). All samples were diluted 1:25 and stored at -20 ºC. The plate setup was designed for sample maximization resulting in all samples run in one plate minimizing run to run variation. The PCR amplification conditions consist of 40 cycles with a limit of detection (LOD) of 35 cycles and primer annealing and elongation at 60 ºC. LOD was set to 35 cycles to ensure specific amplification considering the analytical variation introduced to the analysis by the reverse transcription step[69].

A total of seven genes were investigated including two reference genes, *GAPDH* and *ATCB*. The genes investigated were chosen in connection with protein markers assessed using immunocytochemistry. The selected genes focus on characterizing cells present in the culture after different differentiation timepoints and identification of synaptogenesis and neurite outgrowth formation. *NES*, *GFAP*, and *GAP43* are related to analyzing different neural subpopulations expected in the culture while *MAP2*, *DLG4*, and *SYP* are related to the analysis of synaptogenesis and neurite outgrowth. See Table 3 for primer information.

Table 3. ***Primer information for TaqMan Gene Expression Assay***

Gene	Gene function	Assay ID	Supplier	Catalog number
ACTB	Reference gene	Hs01060665_g1	Thermo Fisher	4331182
DLG4	Synaptogenesis	Hs01555367_m1	Thermo Fisher	4351370
GAP43	Maturing neurons	Hs00967138_m1	Thermo Fisher	4331182
GAPDH	Reference gene	Hs02786624_g1	Thermo Fisher	4331182
GFAP	Astrocytes	Hs00909233_m1	Thermo Fisher	4331182
MAP2	Neurite outgrowth	Hs00258900_m1	Thermo Fisher	4331182
NES	Neural stem cells	Hs04187831_g1	Thermo Fisher	4331182

3.5 Protein marker analysis using Immunocytochemistry

Complementary to gene expression analysis, assessment of possible effects on protein markers related to neurodevelopment following exposure to non-cytotoxic concentrations of AA and GA was implemented. NSCs were split as described in 3.2.1 and seeded with a cell density of 24000 cells/well in black 24-well plates (82406, Ibidi, Germany) in NI medium. After 1 day of proliferation the cells were differentiated and exposed to $1x10^{-5}$ M, $1x10^{-6}$ M, and $1x10^{-7}$ M of the test substances in ND medium for 14 and 21 days, refreshing medium twice per week. A solvent control containing 0.1% sterile H_2O was included. Stock dilutions and medium were prepared as described in appendix 1 and 3.

After differentiation and exposure, the cells were fixed with 4% formaldehyde for 10 min at room temperature and washed twice with Phosphate Buffered Saline (PBS) containing Ca^{2+} and Mg^{2+} (+). Cells were permeabilized with 0.1 % Triton X and 3.5 % bovine serum albumin (BSA) diluted in PBS+. After 15 min permeabilization the cells were incubated in a blocking buffer consisting of 3.5% BSA for a further 15 min, to prevent nonspecific binding of antibodies. After blocking, the cells were incubated with primary antibodies as described in Table 4 at 4 ºC overnight. The cells were stained with primary antibodies related to neurodevelopmental processes in duplicate for each concentration. For analysis of synaptogenesis, the cells were stained with MAP2 (chicken), SYP (rabbit), and PSD95 (mouse). Analysis of neural progression was performed using nestin (rabbit) and Ki67 (mouse) and characterization of neuronal subpopulations present in the culture after different stages of differentiation was achieved by staining with GABA (mouse), TH (rabbit), and MAP2 (chicken). The plates were sealed with parafilm to reduce evaporation during incubation. After incubation, the primary antibodies were recovered and reused up to three times. Next, the cells were washed twice in PBS+ and incubated at room temperature for 1 h with secondary antibodies (Abcam) in dilution 1:500 (Table 5). DAPI was used for nucleus quantification in

dilution 1:1000 (Table 5). Plates were sealed with parafilm and packed in aluminum foil to avoid degradation of the fluorescent marker on secondary antibodies. The cells were then washed twice and stored in PBS+ and sent to the Norwegian Radium Hospital for High Content Imaging (HCI).

Table 4 ***Primary Antibody Information for Immunocytochemistry.***

Protein	Marker Function	Dilution Factor	Supplier	Catalog number
GABA	GABAergic neurons	1:200	Sigma Aldrich	A0310
Ki67	Cell cycle	1:300	Merck	MAB4190
MAP2	Neurite outgrowth	1:500	Abcam	ab5392
Nestin	Neural Stem cells	1:200	Sigma Aldrich	N5413-100UG
PSD95	Synaptogenesis	1:300	Abcam	ab13552
SYP	Synaptogenesis	1:200	Abcam	AB14692
TH	Dopaminergic neurons	1:400	Millipole	AB152

Table 5. ***Secondary Antibody Information for Immunocytochemistry.***

Secondary antibody	Dilution Factor	Supplier	Catalog Number
DAPI	1:1000	Thermo Fisher	62248
Goat Anti-Chicken IgY H&L (DyLight 488) preabsorbed	1:500	Abcam	ab96951
Goat Anti-Rabbit IgG H%L (DyLight 650)	1:500	Abcam	ab96902
Goat Anti-Mouse IgG H&L (DyLight 550) preabsorbed	1:500	Abcam	ab96880

3.5.1 High Content Imaging

Fluorescence microscopic images for later quantification of HCI analysis were performed by Vigdis Sørensen (Phd) from the Norwegian Radium Hospital on their advanced fluorescence microscopy core facility. Immunocytochemistry was visualized by HCI on a Nikon ECLIPSE Ti2-E microscope equipped with CSU-W1 spinning disk confocal unit, Prime BSI sCMOS camera, and 405 nm, 488 nm, 561 nm, and 638 nm lasers. Multichannel images of random fields of view were captured for each well and analyzed using NIS-Element AR Analysis software and/or Fiji/ImageJ. High content images are presented as maximum intensity projections of Z-sections adjusted by linear brightness contrast.

3.6 Statistics

Graphs were prepared using GraphPad Prism version 5. Statistical analysis was performed using JMP Pro 15. Statistical significance was assessed by Steel method as post-test. For all graphs, an asterisk (*) over a bar or bracket indicates a statistically significant difference ($p < 0.05$) with the specified control group. A statistically significant difference of $p<0.01$ or $p<0.001$ is marked with ** and ***, respectively.

3.6.1 ΔΔCq Method

The ΔΔCq Method was used for calculations of relative gene expression for the gene of interest. The average of the quantification cycle (C_q) for three biological replicates was calculated for each condition with CFX Manager software. The average of reference genes, *ACTB* and *GAPDH*, was subtracted from each condition to obtain the ΔCq value. Then the obtained ΔCq value of the control was subtracted from ΔCq of the gene of interest to obtain the ΔΔCq visualized in graphs prepared using GraphPad Prism 5.

4 RESULTS

4.1 Neural Stem Cells in culture

Culture development from undifferentiated NSCs is shown in the phase-contrast imagery presented in Figure 6. As illustrated in Figure 6, and based on subjective impression derived from a visual inspection of a large number of cultures, it appears that the cell density increased slightly as differentiation progressed. The cytoplasmic outgrowth of undifferentiated cells was short and sparse. Upon inducing differentiation, most cells' cytoplasmic outgrowth lengthened possibly indicating outgrowth and more complex neurite network formation. The number of apoptotic cells attached to the top layer of living neurons, seen as bright shrunken cells, increased as the culture developed.

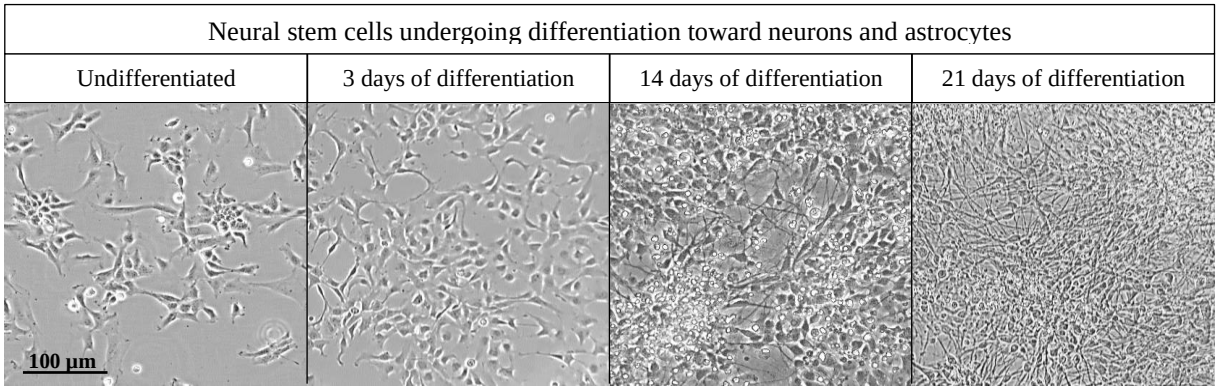


Figure 6. ***NSC undergoing differentiation toward neurons and astrocytes.*** *Phase Contrast Images of undifferentiated NSC and NSC undergoing differentiation for 3, 14 and 21 days in culture (with 10x magnification insets).*

4.2 Alamar Blue™ cell viability assay

4.2.1 Optimization

To examine possible effects of cell density, incubation time and substrate concentration on viability measurements, an optimization of Alamar Blue™ was performed. Development of fluorescence as a measure for cell viability during incubation for up to 8 h with different dilutions of Alamar Blue™ is exemplified in Figure 7. The 1:6 dilution showed a steeper curve with a larger difference in cell viability between exposed and unexposed cells than in the 1:10 dilution.

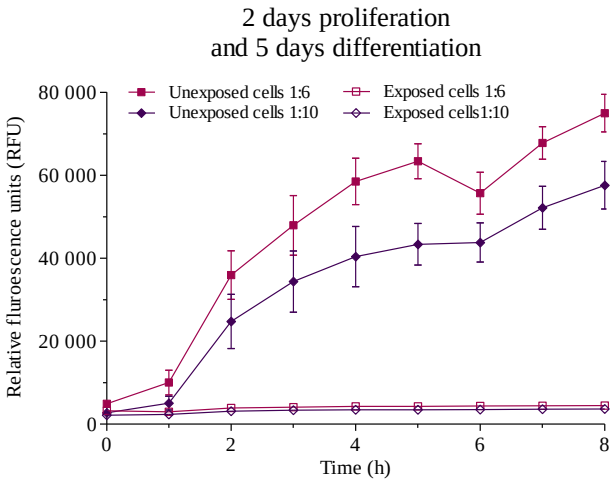


Figure 7. ***Development of fluorescence during incubation with 1:6 or 1:10 dilution of Alamar Blue™ in NSCs proliferated for 2 days and differentiated for 5 days with and without exposure of 1x10^{-3} M GA.***

The reduction of resazurin as a result of cells exposed to 1x10^{-3} M GA versus control after up to 8 h of incubation with Alamar Blue™ is shown in Figures 8 (student project) and 9 (NIPH scientist). Cells exposed for 1x10^{-3} M GA for 1 day (Figures 8 and 9 A and C) revealed a reduction of resazurin at a later timepoint than cells exposed for 3 (Figures 8 and 9 B) and 5 days (Figures 8 and 9 D). Figure 8 A and C show that cells proliferated for 2 days (C) showed a larger decrease in substrate concentration following exposure to 1x10^{-3} M GA than cells proliferated for 1 day (A). For all except cells proliferating for 1 days and differentiating for 1 day, the 1:6 dilution showed a larger reduction of resazurin than 1:10. The cells proliferated for 2 days and differentiated for 1 day showed an overall reduction of below 50% of control after 8 h of incubation for both dilutions (C). In contrast to this, the fluorescence development flattened after 4 and 3 h of incubation for cells differentiated for 3 and 5 days, respectively.

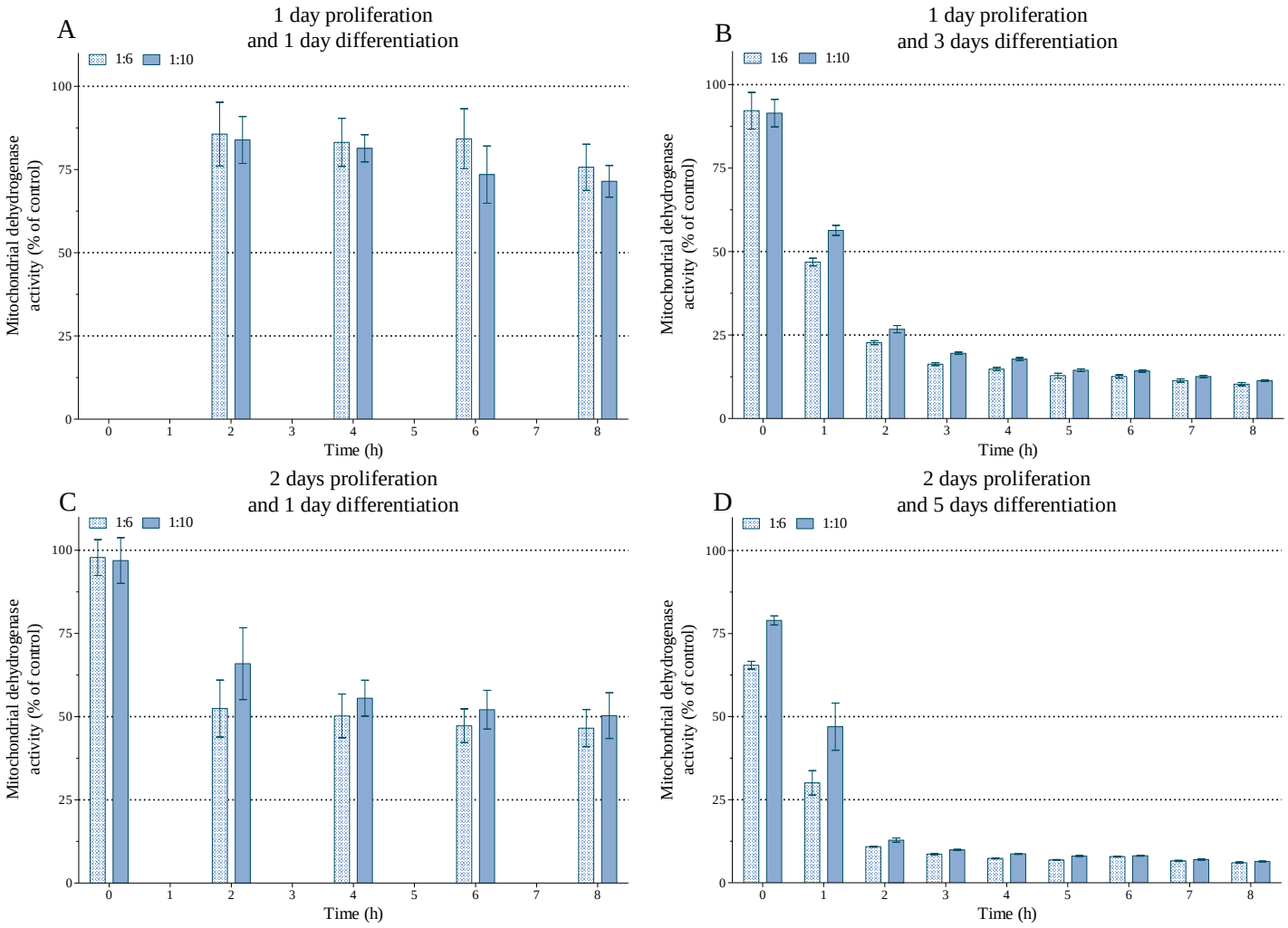


Figure 8. ***Reduction of resazurin by mitochondrial dehydrogenase as a function of incubation time using 1:6 (light blue) and 1:10 (darker blue) dilution of Alamar Blue™ in NSCs following exposure to 1x10^{-3} M GA (student project).*** *All plates were seeded with 7000 cells/well in 96-well plates and proliferated for 1 (A, B) or 2 (C, D) days before starting differentiation and exposure with or without 1x10^{-3} M GA. Cell were exposed while undergoing differentiation for 1 (A, C), 3 (B) and 5 days (D). All data presented as mean ± SD of 12 replicates normalized by control.*

Figure 9 A and C show differences in cell viability having an impact on the reduction of resazurin after 1 day of exposure to 1x10^{-3} M GA. Upon 3 and 5 days of exposure differences in cell densities did not have an observable impact on the viability measurements after 4 h of incubation with Alamar Blue™ (B and D).

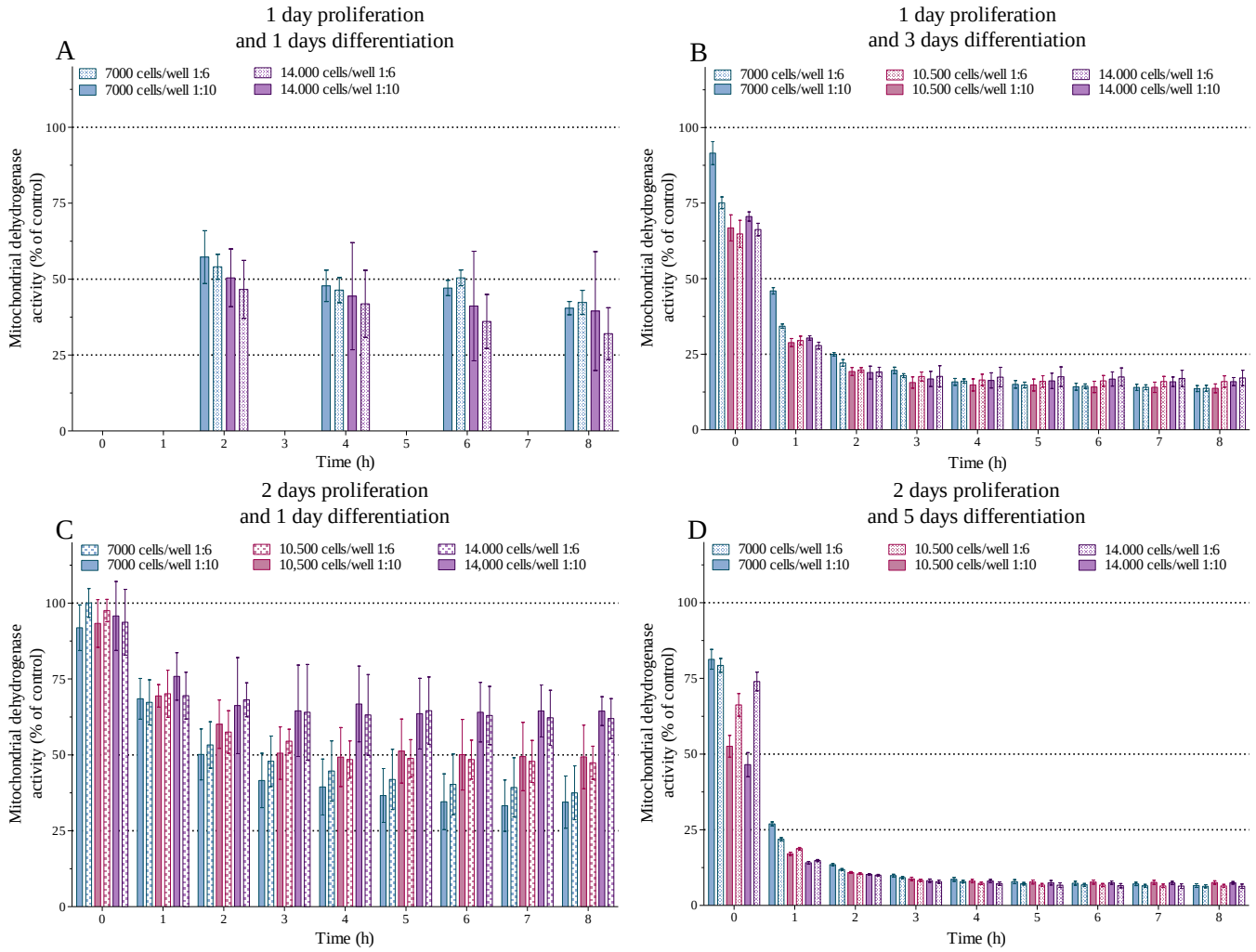


Figure 9. ***Reduction of resazurin by mitochondrial dehydrogenase as a function of incubation time using 1:6 (light colors) and 1:10 (darker colors) dilution of Alamar Blue™ in NSCs following exposure to 1x10^{-3} M GA for different cell densities (trained personnel at NIPH).*** *Cells were seeded with 7000 cells/well, 10500 cells/well or 14000 cells/well in 96-well plates and proliferated for 1 (A, B) or 2 (C, D) days before starting differentiation and exposure with or without 1x10^{-3} M GA. Cells were exposed while undergoing differentiation for 1 (A, C), 3 (B) and 5 days (D). All data are presented as mean ± SD of 12 replicates normalized by control.*

4.2.2 Edge Effect

During incubation, a discrepancy in relative fluorescence units (RFU) output was discovered between the wells on the periphery of the 96-well plate (cell control in a vertical column) and the wells placed inside the plate (cell control in a horizontal row) (Figure 10). To exclude the possibility that the variations observed were due to culturing conditions unexposed cells were seeded at the periphery of the plate and compared to unexposed cells placed inside the plate. As seen in A, the cell control placed along the periphery (vertical cell control) had a lower RFU than the cell control surrounded by wells (horizontal cell control). The data from A was performed by a fellow student. By framing the entire plate with PBS to avoid potential artifacts due to liquid evaporation the differences between the vertically and horizontally placed cell controls were gone (B). The discrepancy affecting the wells placed at the periphery of the plate was also counteracted by normalizing the data from edge wells to the mean of control wells also placed at the periphery of the plate.

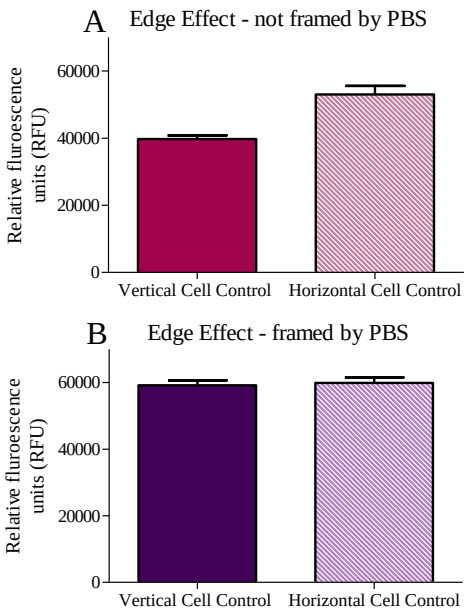


Figure 10. ***Edge effect in NSCs****. Relative fluorescence units of cell control vertically placed at the periphery of the 96-well plate (dark red) and horizontally inside the plate (light red) (A). Data collected by fellow student. Relative fluorescence units of cell controls placed vertically (dark purple) or horizontally (light purple) in a 96-well plate entirely framed with PBS (B). Repeated experiment with data collection by me.*

4.2.3 Effects of acrylamide and glycidamide on viability in NSCs undergoing differentiation

The possible cytotoxic impact of AA and GA on NSCs undergoing differentiation was assessed by measuring mitochondrial dehydrogenase activity with Alamar Blue™ cell viability assay. Different durations of exposure were used to reflect a potential for vulnerability in the differentiation process and a possible accumulation effect.

The percentage reduction of resazurin in cells exposed to AA and/or GA after 3 h 30 min incubation with 1:10 dilution of Alamar Blue™ is shown in Figure 11. AA and GA significantly reduced cell viability in a time- and concentration-dependent manner. Statistically significant reduction of resazurin was achieved after 1 day of exposure to the highest concentration of AA and/or GA tested (A, E, and I). GA was the most cytotoxic with a statistically significant reduction of resazurin for concentrations ranging from $1x10^{-4}$ M to $3x10^{-3}$ M after continuous exposure for 14 days (G). Combined exposure to $1x10^{-5}$ M AA and GA for 21 days resulted in a statistically significant decrease in cell viability (L).

Cell viability significantly increased after 1 day of exposure with lower concentrations followed by a decrease after 3 and 14 days before stabilizing around control levels after 21 days of continuous exposure. The largest increase in cell viability was seen for combined exposure to AA and GA with an up to 32% increase in mitochondrial activity after 1 day (I). When compared to NSCs differentiated for 1 day, cell viability significantly decreased following continuous exposure to $1x10^{-5}$ M, $1x10^{-6}$ M, and $1x10^{-7}$ M of AA and the combination for 14 and 21 days (supplementary statistics illustrated in a table in appendix 4). After 3 days of exposure, no statistically significant modifications in cell viability were detected for NSCs exposed to $1x10^{-5}$ M, $1x10^{-6}$ M, and $1x10^{-7}$ M of AA and/or GA, except for cells exposed to $1x10^{-5}$ M AA, when compared to cells exposed for 1 day (appendix 4).

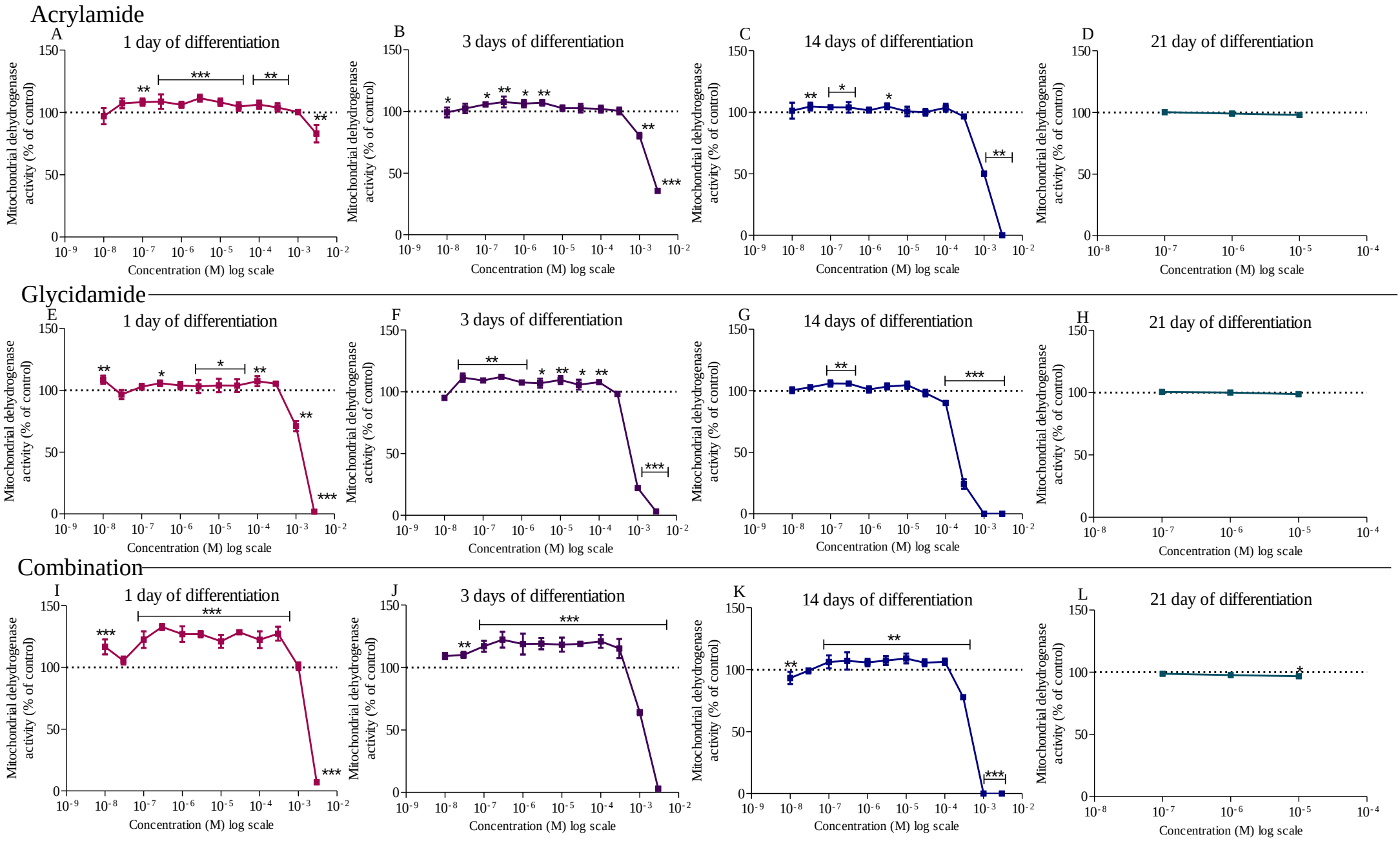


Figure 11. ***Effects of AA and GA exposure on cell viability for NSCs undergoing differentiation assessed by Alamar Blue™ assay.*** *All plates were seeded with 7000 cells/well in 96-well plates and proliferated for 1 (C, D, G, H, K, L) or 4 days (A, B, E, F, I, J) before starting differentiation and exposure to AA and/or GA. NSC were differentiated and exposed to twelve concentrations ranging from $3x10^{-3}$ M to $1x10^{-8}$ M of AA (A-D), GA (E-H) or a combination of the two (I-L) for 1 day (A, E, I) 3 days (B, F, J), 14 days (C, G, K) or 21 days (D, H, L). All data presented as mean ± SEM of three experiments normalized to solvent control. *p<0,05, **p<0,01, ***p<0,001 compared to unexposed solvent control, obtained by Steel method.*

4.3 Gene expression analysis using real time polymerase chain reaction

As a part of an ongoing internal characterization as well as of the establishment of the NSC model at NIPH, gene expression of markers related to neurodevelopment in NSCs undergoing differentiation for up to 28 days was assessed. Expression of *MAP2* during the differentiation process from undifferentiated cells toward more differentiated neurons and astrocytes for up to 28 days is presented in Figure 12. The development of *MAP2* expression during differentiation was as expected with a statistically significant increase in expression as neurons matured. Upon 7 days of differentiation a 2-fold change increase in *MAP2* expression was observed whereas differentiation for 28 days resulted in a 4-fold change increase.

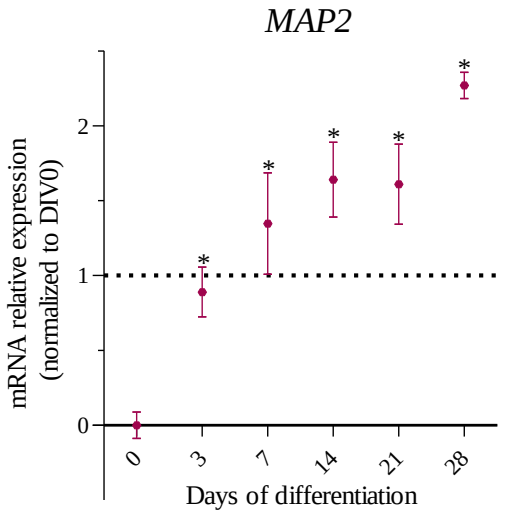


Figure 12. ***Expression of MAP2 in unexposed NSCs undergoing differentiation for up to 28 days.*** *Results from an ongoing internal characterization at NIPH performed by trained personnel. Data presented as mean ± SEM normalized to undifferentiated cells. DIV0 = undifferentiated cells. *p<0.05 compared to undifferentiated cells, obtained by Steel method.*

Five genes related to neurodevelopment were assessed for possible gene expression alterations on NSCs undergoing differentiation induced by exposure to non-cytotoxic concentrations of AA and GA. mRNA expression levels of four genes after continuous exposure to $1x10^{-5}$ M, $1x10^{-6}$ M, and $1x10^{-7}$ M of AA and GA for 3, 14, and 21 days are presented in Figure 13. One gene, *GFAP*, failed to show amplification within the LOD set for our real-time PCR analysis. There were no statistically significant alternations in gene expression compared to control for any concentrations or combinations for the time points investigated.

Expression of *NES* showed a minor concentration-dependent upregulation compared to control for cells exposed to AA and/or GA with the most apparent trend seen in the combination after 3 days of exposure (A). The highest concentration of combined AA and GA tested, resulted in a non-statistically significant fold change of 2. Exposure for 14 and 21 days resulted in an apparent downregulation of the neural stem cell marker.

Minor upregulation of *GAP43* compared to control was observed for the higher concentrations of AA following exposure for 3 days, whereas little effect was found after 14 and 21 days (B). A marginal increase in *GAP43* expression resulting from 3 days of exposure to $1x10^{-7}$ M GA could be detected followed by a marked decrease after 14 days. An apparent concentration-dependent increase in *GAP43* expression was seen following exposure to AA combined with GA for 3 days with a fold change of 2. However, this possible increase was not observed after continuous exposure for 14 and 21 days.

No effects were observed after 3 days of AA exposure on *MAP2* expression whereas a minor downregulation could be perceived for the later time points (C). Exposure to $1x10^{-7}$ M GA resulted in an upregulation after 3 days followed by a marked decrease in *MAP2* expression after 14 days of exposure. Combined AA and GA exposure for 14 days resulted in a downregulation of *MAP2* whereas 3 and 21 days exposure showed a similar trend with the lowest concentration resulting in downregulation and the highest resulting in an upregulation compared to control. However, exposure to $1x10^{-5}$ M AA combined with GA resulted in a 2-fold change increase.

A modest increase in *DLG4* expression as a result of exposure to AA and/or GA was observed for the NSCs undergoing differentiation for 3 days whereas exposure for 14 days resulted in a slight downregulation of the synaptogenesis marker (D). A subtle upregulation of *DLG4* after 21 days of exposure to AA was detected whereas exposure to GA and the combination showed a similar decreasing trend.

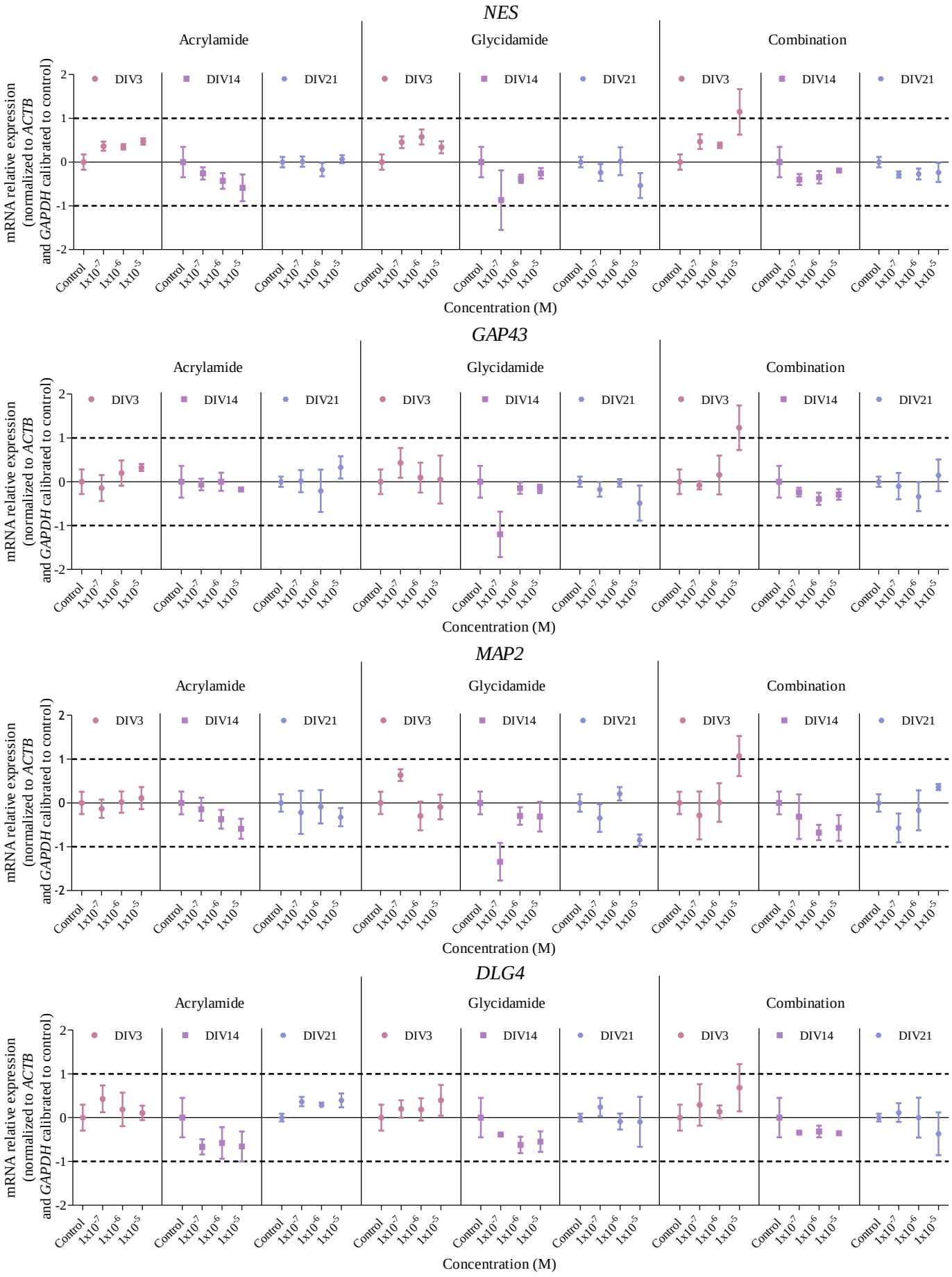


Figure 13. ***mRNA relative expression of four genes related to neurodevelopment after combined differentiation and exposure to AA and/or GA for 3 (pink), 14 (purple) and 21 (blue) days.*** *Expression of NES (A), GAP43 (B), MAP2 (C) and DLG4 (D) were normalized to ACTB and GAPDH and calibrated to unexposed control (ΔΔCq method). All data presented as mean ± SEM of three experiments. DIV = days of differentiation and exposure. Statistics was performed using Steel method.*

4.4 Immunocytochemistry

Figures 14 and 15 show examples of immunocytochemical visualizations of protein markers related to neurodevelopment in NSCs undergoing differentiation (see Table 4) with or without exposure to $1x10^{-5}$ M AA and/or GA for 14 and 21 days using high content imaging (HCI). Quantification of protein markers was not performed and alterations in protein expression can therefore not be determined.

Figures 14 and 15 A visualize the neural stem cell marker nestin localized in the cytoskeletal structure and the cell cycle marker Ki67 localized in the nucleus. Neurite outgrowth was visualized in green by microtubule-associated protein 2 (MAP2) expression, distributed in the cell body and dendrites (Figures 14 and 15 B and C). Presynaptic and postsynaptic neurons were envisioned by the presence of Synaptophysin (SYP) and Post-Synaptic Density protein 95 (PDS95) (Figures 14 and 15 B), respectively, whereas GABAergic and dopaminergic neurons

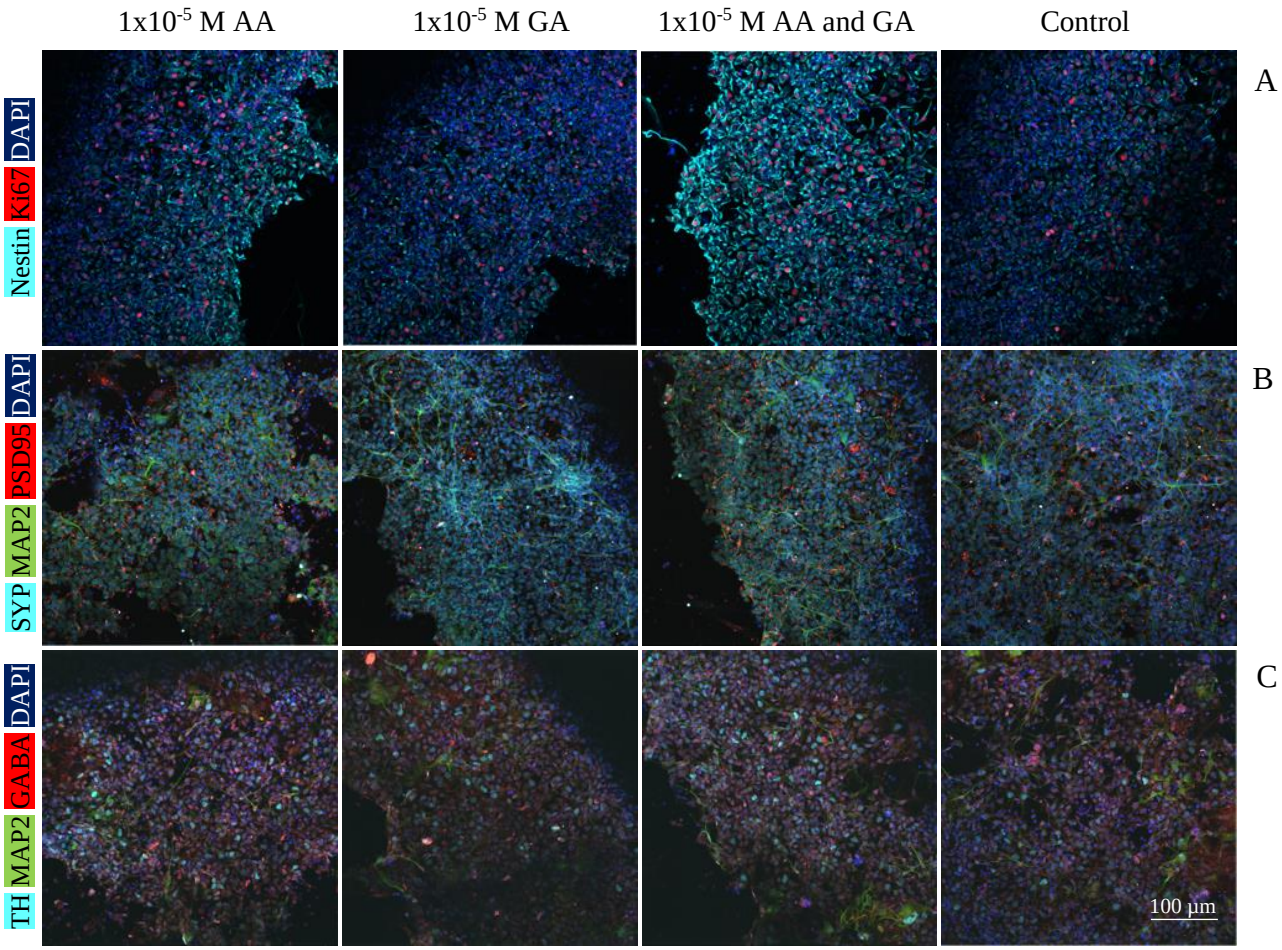


Figure 14. ***Qualitative immunocytochemical images of NSC protein markers related to NSCs, differentiation, neurite outgrowth and synaptogenesis upon exposure to AA and GA for 14 days****. Immunocytochemical images of nestin (turquoise) and Ki67 (red) (A), of PSD95 (red), MAP2 (green) and SYP (turquoise) (B) and of GABA (red), MAP2 (green) and TH (turquoise) (C) in NSCs undergoing differentiation with or without exposure to $1x10^{-5}$ M AA and/or GA for 14 days. DAPI (blue) was used for visualization of nucleuses.*

were visualized by gamma- aminobutyric acid (GABA) and Tyrosine hydroxylase (TH), respectively (Figures 14 and 15 C).

The protein markers were detected in control cells as well as for cells exposed to AA and/or GA for 14 and 21 days (Figures 14 and 15). Some visible increase in fluorescence intensity of nestin and Ki67 could be observed when comparing cells exposed to AA and the combination for 14 days to control (Figure 14 A). However, no apparent visible alternations in fluorescence intensity of markers related to synaptogenesis and neural differentiation following exposure to AA and/or GA for 14 days were observed when compared to control (B and C).

Upon 21 days of differentiation and exposure, no apparent visible difference in fluorescence intensity of nestin and Ki67 nor intensity of the synaptic markers was observed when comparing to control (Figure 15 A and B). A minor difference in intensity of TH could be observed when comparing NSCs exposed to GA for 21 days to control (C). Increased neurite outgrowth, as well as a more complex network formation, could be observed after 21 days when comparing to cells differentiated for 14 days. This was visualized by a perceived higher expression of MAP2 (green) and SYP (white/turquoise) in the cell body, dendrites, and the presynaptic terminal after 21 days when compared to cells differentiated for 14 days (Figures 14 and 15 B).

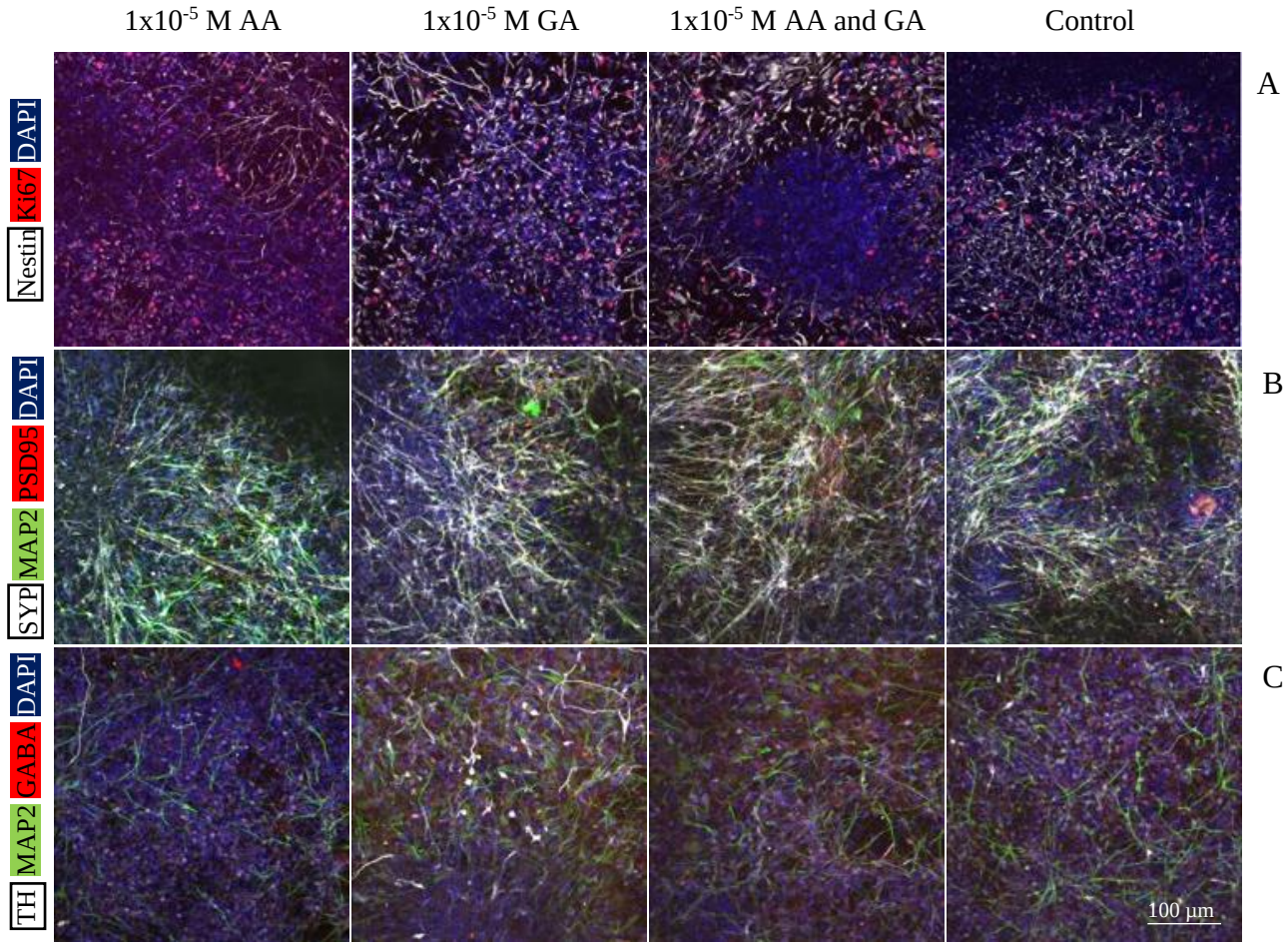


Figure 15. ***Qualitative immunocytochemical images of NSC protein markers related to NSCs, differentiation, neurite outgrowth and synaptogenesis upon exposure to AA and GA for 21 days****. Immunocytochemical images of Nestin (white) and Ki67 (red) (A), of PSD95 (red), MAP2 (green) and SYP (white) (B) and of GABA (red), MAP2 (green) and TH (white) (C) in NSCs undergoing differentiation with or without exposure to 1x10-5 M AA and/or GA for 21 days. DAPI (blue) was used for visualization of nucleuses.*

5 DISCUSSION

Fetal dietary exposure to AA and GA has been shown by the presence of hemoglobin adducts in umbilical cord blood. The presence of AA and GA in umbilical cord blood revealed that the placenta provides limited protection of the fetus to exposure to AA and GA originating from maternal diet. AA is also detected in human breast milk indicating that fetal neurodevelopment could be influenced by exposure originating from maternal diet both prenatally and postnatally. Although prenatal exposure to AA has been negatively associated with fetal growth, little is known about its implication on human neurodevelopment and in particular cognitive development[9]. Therefore, due to its widespread dietary exposure in the general population, DNT is of concern. Severely restricted fetal growth is strongly linked to neurodevelopmental deficits[31], and it is, therefore, an urgent need to assess the possible effects of fetal AA and GA exposure on neurodevelopment.

In this thesis, an assessment of the possible cytotoxic impact of AA and GA on NSCs undergoing differentiation, as well as possible perturbations of key neurodevelopmental processes like differentiation and synaptogenesis was performed using viability measurements, gene expression, and protein markers. Our results revealed that human-relevant concentrations slightly increased cell viability in NSCs undergoing differentiation, with the most apparent effect on immature neurons. Millimolar concentrations decreased cell viability by inducing cell death in a time- and concentrations-dependent manner. The results suggested that GA was more potent than AA and that combined exposure resulted in an additive effect.

Gene expression analysis of three non-cytotoxic, human-relevant concentrations revealed only minor changes, although some concentration-dependent trends could be observed. Expression of some genes showed slight alterations close to 2-fold changes, although not statistically significant. The findings of human relevance are for most genes considered to be a 2-fold change difference[70], although it could be argued that lower trends also may be of some relevance.

Immunocytochemical visualization of protein markers revealed a qualitative protein expression in line with previous characterizations[3]. Phase-contrast microscopy of the NSCs during differentiation revealed that the culture developed morphologically as expected. The use of NSCs derived from hiPSC is highly relevant in human DNT testing as they do not exhibit tumor-growth-related responses to chemical exposures nor adult brain physiology as well as avoiding interspecies differences[51].

5.1 Neural stem cells differentiating into a mixed culture of neurons and astrocytes

The culture was visually inspected during the course of differentiation to ensure that the cells developed as expected, and thereby ensure more reliable results. As seen in Figure 6 there is minor observable difference between undifferentiated NSCs, and cells differentiated for 3 days apart from possibly seeming to start connecting with nearby neurons. Upon differentiation, the network formation becomes more extensive and corresponds with phase-contrast imaging published in a previous study from EC-JRC[57], from which the model was transferred. The number of apoptotic cells in the culture increased with differentiation. Apoptosis is a naturally occurring event in neurodevelopment and would be discarded by immune cells *in vivo*[32]. This process is not replicated *in vitro,* and apoptotic cells are therefore visible as bright shrunken cells attached to living neurons. By visually evolving as expected and in line with EC-JRCs characterization, further results acquired by the use of this model is considered reliable.

5.2 Optimization of Alamar Blue™

An optimization of Alamar Blue™ cell viability assay was performed to investigate the possible effects of cell densities, incubation time and substrate concentration on cell viability by comparing commercial protocols from Invitrogen[64] and Promega[65] used at NIPH and EC-JRC, respectively. The optimization was done once for indication and all conclusions are based on visual inspection only of the data. Conclusions are mainly based on results provided by the validation done at NIPH due to inexperience with cell cultivation which resulted in varying cell densities and less reliable results from the student project.

As seen in Figure 9 the development of fluorescence stabilizes after around 3-4 h of incubation with Alamar Blue™ for every cell density at every time point investigated. The different cell densities were used to simulate differences in confluency during an experiment with 7000 cells/well proliferating for 1 day representing the cell density recommended for use in experiments[3] and 14000 cells/well proliferating for 2 days representing a fairly confluent well. After 1 h incubation, there was an observable difference in viability when comparing 7000 cells/well and 14000 cells/well with 1:10 dilution of Alamar Blue™ in which is not present after 3-4 h incubation (Figure 9 B and D). Incubation for 1 h could therefore result in a false difference in viability caused by a difference in confluency and an incubation time of 3-4 h was chosen for further analysis of cell viability.

As shown in Figures 8 and 9, and confirmed by visual inspection of plates after Alamar Blue™ cell viability assay, shorter exposure to $1x10^{-3}$ M GA affected the cells negatively but did not result in complete cell death (Figures 8 and 9 A and C). Exposure for 3 and 5 days results in around 80% cell death for all cell densities (Figure 9 B and D). There was less cell death observed for the higher cell densities for the cells proliferated for 2 days and differentiated for 1 day when comparing with the lower densities (Figure 9 C). This corresponds to the finding of Wu et al.[71] (2020), who found that there was a negative correlation between cytotoxicity and higher cell densities in their experiment. Based on this, it can be speculated that higher cell numbers may protect against cytotoxic compounds up to a point.

Figure 7 shows a larger difference between exposed and unexposed cells in fluorescence development after incubation with 1:6 dilution of Alamar Blue™ compared with 1:10 dilution. However, when data were normalized to control this difference between 1:6 and 1:10 dilution was reduced (Figures 8 and 9) and almost negligible. The 1:10 dilution was concluded to be adequately sensitive for our cell model, clearly differentiating between living and dead cells. An Alamar Blue™ dilution of 1:10 and an incubation time of 3-4 h were chosen for further experiments.

5.3 Influence of acrylamide and glycidamide on cell viability

The influence of AA and GA on cell viability was investigated to assess the possible cytotoxic impact of chemical exposure on NSCs undergoing differentiation and the possible occurrence of cell death. As seen in Figure 11 exposure to low concentrations of AA and/or GA resulted in a statistically significant increase in mitochondrial dehydrogenase activity when compared to control, with the most apparent increase seen in immature neurons. The most apparent increase in cell viability resulted from short-term exposure to AA combined with GA. This is in accordance with the findings of Attoff et al.[26] (2016), reporting a similar increase in SH-SY5Y cells after exposure with AA in the same concentration range. The results indicate that the increase in viability was due to increased proliferation rather than increased metabolic activity. In another paper by Attoff et al.[27] (2020), they found that low concentration of AA attenuated differentiation by sustaining proliferation and reducing neurite outgrowth in SH-SY5Y cells. Interestingly, this implies that the increase in fluorescence observed, most probably was due to an increased proliferation of NSCs possibly combined with an attenuated differentiation process. An increased number of neurons have been implicated in neurodevelopmental disorders like Autism Spectrum Disorders (ASD)[72]. In one study children

with ASD were found to have 67% more neurons in the prefrontal cortex than in the control group[72].

Cytotoxicity upon exposure to AA and GA was detected in a time- and concentration-dependent manner. Visual inspection of plates revealed the decrease in resazurin to be caused by cell death rather than metabolic inactivity. A previous study by Chen and Chou[28] et al. (2015) found that exposure to high concentrations of AA attenuated proliferation in SH-SY5Y cells in a time- and concentration-dependent manner. Park et al[29]. (2010) concluded that exposure to high concentrations of AA significantly affected the proliferation and viability of C17.2 NPCs through oxidative stress and ROS generation. Millimolar concentrations of AA were also found to induce both necrotic and apoptotic cell death[29]. The decrease in fluorescence observed for the highest concentrations caused by cell death could be due to induction of apoptosis and/or necrosis and reduced proliferation following exposure to AA and GA. Although an excessive number of neurons in the infant brain has been implicated in the development of ASD[72] excessive apoptosis in childhood and adolescence has also been observed in children with ASD[73]. Disturbed apoptosis could influence normal brain development and maturation and possibly mediate symptoms of ASD[73]. Binding of chemicals to thiol-groups on proteins involved in protection against oxidative stress during brain development has been proposed in a recent adverse outcome pathway (AOP) to contribute to impairment of learning and memory (under development and not yet endorsed by OECD)[74]. The evidence in this AOP is mainly built on data from methylmercury exposure but is also relevant for AA due to its thiol binding properties[9]. It could be speculated that millimolar concentrations of AA and GA that trigger cell death/injury, in turn, could lead to decreased network formation implicated in the AOP network for impairment of memory and learning. However, more research is needed before these speculations are substantiated.

The time- and concentration-dependent cytotoxicity observed after exposure to AA and GA became most apparent when comparing maturing neurons to their nearly undifferentiated counterparts. NSCs differentiated and exposed for 14 and 21 days showed a statistically significant decrease in viability when compared to NSCs differentiated and exposed for 1 day. After 21 days of exposure, the observed effects of AA and GA on cell viability were reversed to control levels. Mature neurons are post-mitotic cells that are believed to possess mechanisms in which enhance survival and resist apoptotic stimuli[75]. In contrast to this, our results indicate that the more matured neurons were more sensitive to the cytotoxic effects induced by chemical exposure resulting in an observed reduction in cell viability when compared to nearly

undifferentiated cells exposed to the same concentration. Both AA and GA are water-soluble with distribution to most organs in the human body including the brain. Both AA and GA are detoxified and almost completely excreted through the kidneys *in vivo* and no bioaccumulation is reported for AA[9]. This detoxification pathway is not simulated in our NSC model leading to a continuous exposure possibly not representative for human dietary exposure. It could be speculated that the induced sensitivity to cytotoxic effects caused by chemical perturbance results from an accumulating effect in the NSC model in which may not be replicated *in vivo*.

When comparing the cytotoxic effects of AA and GA it becomes clear that GA causes a decrease in cell viability for NSCs at lower concentrations than AA. Upon 1 day of exposure to $1x10^{-3}$ M GA, there was a 30% decrease in viability in contrast to AA which did not cause any difference from control. The same tendency can be observed after 3 days of exposure where $1x10^{-3}$ M caused an 80% decrease in viability whereas AA caused around 20%. These results suggest that GA is more potent than AA and possibly perpetuates the cytotoxicity observed when combined with AA. While both AA and GA bind to thiol groups on cellular proteins possibly important in neural function GA also forms adducts with DNA which is speculated to be the cause of genotoxic and carcinogenic properties associated with AA exposure[8]. It is tempting to speculate that GA is more potent than AA due to this reactivity with DNA. Due to the experimental design, it is difficult to specify the additive effect observed when combining AA and GA. Whereas $1x10^{-3}$ M AA and GA caused 20% and 80% decrease in viability, respectively, $1x10^{-3}$ M AA combined with GA caused a 35% reduction in viability. As described in 3.3.2.1 the combination was made by mixing master stocks of AA and GA with a given concentration 50/50 to create a combination with a similar total concentration to the AA and GA master stocks. This resulted in the concentrations of AA and GA individually being half of the total concentration in the combination. When taking this into account the individual concentrations of AA and GA in the combination with a total concentration of $1x10^{-3}$ M would be $5x10^{-4}$ M which is closer to exposure to $3x10^{-4}$ M of AA and GA. Upon 3 days of exposure to $3x10^{-4}$ M of AA and GA, there were no or only minor changes in viability observed on NSCs. Based on this it is tempting to speculate that the effect of AA combined with GA may act synergistically inducing cytotoxicity. However, further studies are needed with a more appropriate experimental design for the determination of the effects or interactions (synergistic or antagonistic) of exposure to AA combined with GA.

5.4 Background for choice of concentrations used for further analysis of gene expression and protein markers

Assessment of possible cytotoxic properties of AA and GA on NSCs undergoing differentiation was also done to enable the selection of non-cytotoxic concentrations for further analysis of potential alterations in gene expression and/or protein markers relevant to neurodevelopment. Combined exposure and differentiation for 3, 14, and 21 days was investigated further for the potential impact of AA and GA exposure on gene and protein markers. As seen in Figure 11 longer exposure induces more cytotoxicity with possible incipient cytotoxicity for $1x10^{-4}$ M and $3x10^{-5}$ M in NSCs exposed to GA for 14 days. To ensure sufficient RNA output for gene expression analysis $1x10^{-5}$ M was chosen for the highest concentration because, after 14 days of exposure, there were no sign of incipient cytotoxicity. Further, $1x10^{-6}$ M was chosen for the intermediate concentration and $1x10^{-7}$ M was chosen to be the lowest concentration. Daily exposure of dietary AA is estimated to result in $2\text{-}10x10^{-9}$ M unbound AA in plasma and both AA and GA have a high placental transfer[9]. The lowest concentration selected, $1x10^{-7}$ M, is the closest to believed human exposure with $1x10^{-8}$ M being the estimated daily exposure for adult high consumers. The placenta provides limited protection of the fetus with multiple *ex vivo* studies showing AA and GA in fetal and maternal blood reaching an equilibrium[9]. With AA reaching breast milk, breastfeeding infants continue AA exposure originating from maternal diet even after birth. AA is also detected in infant formula and porridge resulting in dietary exposure even after infants stop breastfeeding. Thus, the human brain may be continuously exposed to AA (and possibly GA) for the entire fetal development even continuing after birth which is a cause for concern. Few studies have investigated the possible relationship between prenatal exposure to AA and DNT effects although there have been studies associating prenatal dietary exposure of AA through maternal diet and reduced fetal birth weight and head circumference[9, 19, 30]. There is a need for assessment of DNT markers after exposure to AA and GA concentrations mimicking real-life dietary exposure in a model reflecting early brain development in humans. Thus, $1x10^{-5}$ M, $1x10^{-6}$ M, and $1x10^{-7}$ M were chosen for analysis of gene and protein markers related to neural development in a human-relevant NSC model[76].

5.5 Effects of acrylamide and glycidamide on gene expression

Gene expression of *MAP2* on NSCs undergoing differentiation for up to 28 days was assessed as a part of the establishment of the NSC model at NIPH. *MAP2* is used as a marker for mature neurons with an expected increase in expression as neurons mature[37]. A statistically significant, progressive increase in *MAP2* expression from undifferentiated cells was observed with an

increase close to 2-fold change upon 3 days of differentiation (Figure 12). After 28 days of differentiation, there was a 4-fold change increase of *MAP2* expression from the undifferentiated cells. The results indicate that the model differentiates as expected and substantiates that the gene expression results are reliable.

Effects of non-cytotoxic concentrations of AA and GA on gene expression markers related to neurodevelopment in NSCs undergoing differentiation were not statistically significant and possible of low biological relevance. The differences in gene expression from control were mostly less than 2-fold change with considerable variation between the experiments (Figure 13). There are some trends within the time points investigated, however, these changes vary between the time points and are thus not easy to interpret.

Gene expression analysis of *NES* revealed that upon 3 days of exposure to AA and GA the NSC marker showed an increasing trend most apparent in the combination with a 2-fold change expression for the highest concentration. This is in concordance with AA and GA's influence on cell viability where the most apparent increase was seen for the combination as well. This increase in *NES* expression is in support of the argument that the increase in viability observed could be the result of increased proliferation of immature NPCs.

The same concentration-dependent increase can be seen in all genes tested upon 3 days of exposure to the combination. The highest concentration tested resulted in a 2-fold change upregulation in *GAP43* and *MAP2* as well as a minor upregulation of *DLG4*. *GAP43* and *DLG4* are associated with neurite outgrowth, synaptogenesis, and neurodevelopment[38, 44] and the increased expression could possibly be explained by increased differentiation induced by AA and GA exposure. Increased expression of *MAP2*, a microtubule protein mainly expressed in neurons, is associated with neural differentiation[37] but could also be increased due to a higher cell number. Taken together this upregulation upon 3 days of exposure may indicate an increased proliferation as the result of chemical exposure in which did not affect the differentiation process. However, this would not be in concordance with previous studies showing AA sustaining proliferation by attenuating differentiation[26, 27, 77]. Impaired differentiation was determined based on the affected expression of a different set of genes in SH-SY5Y cells[27]. SH-SY5Y is a neuroblastoma cell line chemically induced to differentiate into cells expressing a neuron-like phenotype[26]. Cancer cell lines may still express tumor-growth-related genes possibly affecting cellular responses to chemical exposure[51]. One explanation for the deviating results could be that our cell model is non-cancerous and display a more immature starting point than SH-SY5Y in which could have affected the results. Our

cell model differentiates into neurons and astrocytes in contrast to SH-SY5Y which could also explain the results.

There were no statistically significant modifications in gene expression influenced by exposure to AA and/or GA and only a few concentrations resulted in a 2-fold change expression. A common strategy to assess the biological relevance of differentially expressed genes is the use of 2-fold change and/or p-value thresholds[70]. However, small changes in gene expression (<2-fold change) could also provide biologically relevant information and should be taken into consideration. The developing CNS is particularly vulnerable to chemical disturbances[52] and even minor variations in expression may be of importance. It can also be argued that alterations in gene expression following exposure to AA and/or GA may occur in a different time window than those investigated in this experiment. Gene expression analysis was performed on NSCs undergoing differentiation at the earliest, 2 days after the last medium change containing chemical exposure. It can be speculated that the substances may have only transiently influenced the gene expression and therefore was not detected by this experimental design. In a potential follow-up study, further experiments investigating gene expression shortly after exposure to AA and/or GA on NSCs undergoing differentiation should be included.

5.6 Immunocytochemical visualization of protein markers related to neurodevelopment

Neurodevelopmental protein markers were visualized by immunocytochemistry and HCI to qualitatively assess the overall protein expression in our cell model after chemical exposure. The protein markers were detected in all cells, regardless of exposure and duration, indicating that exposure to AA and/or GA did not interfere with the overall expression of the protein markers investigated. Quantitative differences in protein expression when comparing different exposures could, however, not be determined since the HCI method was not yet completely developed, and only qualitative data were therefore available for visual comparisons.

After 21 days of differentiation, the culture has retained the presence of NSCs still dividing (Figure 15 A). However, the presence of GABAergic and dopaminergic neurons as well as network formation and neurite outgrowths indicate concurrent differentiation into more mature neurons (C). An apparent visible difference in the complexity of network formations can be observed when comparing NSCs differentiation for 14 and 21 days (Figures 14 and 15). These observations are in agreement with a previous characterization of the NSC model where a difference in expression of markers related to neural progression and maturation between 14

and 21 days of differentiation was detected[36]. Previous quantitative evaluations of NSCs undergoing differentiation describe a modest NSC population still present after 21 days of differentiation as well as glutamatergic, GABAergic, dopaminergic neurons, and glial cells[3].

A visible difference in nestin and Ki67 fluorescence intensity in NSCs exposed to AA and the combination for 14 days as well as in TH for cells exposed to GA for 21 days when compared to control was observed (Figures 14 A and 15 C). This could be explained by the method of how the qualitative images were produced. Each image is presented as one Z-section of the NSCs in one well, meaning that the images are not necessarily taken in the same plane. This in turn can be misinterpreted as differences in protein expression. Qualitative images could therefore be argued to be less reliable for comparison when not supported by additional quantitative data. The method is under development at the Norwegian Radium Hospital in collaboration with NIPH and to this date, only the qualitative part of the analysis has been established. Upon successful establishment of the method, advanced analysis can be used to extrapolate quantitative data from the images presented in this thesis. Potentially, quantitative data could reveal differences in protein expression and thereby effects on neurodevelopmental processes upon exposure to AA and/or GA versus control.

The visible difference in nestin and Ki67 intensity in NSCs exposed to AA combined with GA could be related to the increase in viability observed for NSCs exposed for 14 days (Figure 11 K). NSCs exposed to AA combined with GA showed a statistically significant increase in cell viability for $1x10^{-5}$ M, $1x10^{-6}$ M, and $1x10^{-7}$ M after 14 days of exposure. As previously mentioned, exposure to low concentrations of AA has been shown to increase proliferation and attenuate differentiation in SH-SY5Y cells[27]. Attenuated differentiation as the result of increased proliferation could possibly be observed in immunocytochemical images as an increased presence of neurons still in active cell division.

The expression of protein markers was as expected and in concordance with previous quantitative as well as qualitative characterizations of the model[35, 36, 57]. Upon 21 days of differentiation, the NSC model was expected to express approximately 15-20% GABA (GABAergic neurons), 13-20% TH (dopaminergic neurons) as well as retaining 20-30% expression of nestin (NSCs) of the total cells in culture (visualized by DAPI staining)[3]. Some user-dependent variability may although be observed[3]. The expression of Ki67 was also expected to decrease with differentiation while expression of SYP and PSD95 was expected to increase with network formation and synaptogenesis[35, 57]. A GFAP expression of 10-15% as

well as vesicular glutamate transport 1 (VGlu1), indicative of glutamatergic neurons, of 35-40% was also expected upon 21 days of differentiation[3], although not investigated in this thesis.

5.7 Discussion of weaknesses in regard to the model and experiment design

In this thesis, no expression over LOD of *GFAP* was detected, even in unexposed controls. Expression of *GFAP* increases with maturation in the human brain and is most abundant in adults[33]. Nevertheless, a progressive increase of *GFAP* mRNA expression for NSCs differentiating for up to 21 days has been shown during characterization of this cell model[3]. The model's ability to differentiate into mixed cultures of neurons and astrocytes makes it a relevant tool when investigating chemically induced effects on key neurodevelopmental processes including proliferation and differentiation[3]. A previous study performed on this model showed that Nrf2, a protein regulating a cellular defense mediating cytoprotection against oxidative stress, had a more stable expression in astrocytes when compared with neurons and astrocytes were less vulnerable to cell death induced by oxidative stress[3]. Astrocytes have a range of vital functions in the human brain, including providing structural and metabolic support, regulating synapse formation and production of neurotropic factors important for the development and survival of neurons[78]. Astrocyte dysfunction has been linked to neurological disorders like Parkinson's disease[78], schizophrenia[79], and bipolar disease[80]. The presence of astrocytes in our model is essential for its application in DNT testing and possibly for the normal development and function of neurons in our culture. This discovery is in support to preliminary gene expression results from the ongoing characterization at NIPH of the NSC model undergoing differentiation. However, in contrast to the gene expression results, GFAP protein expression has been detected using immunocytochemistry and HCI. This suggests that the gene expression of *GFAP* is rather low in this model at the timepoints investigated, and that protein expression may be more suitable for the detection of astrocytes in culture. Further studies of gene and protein expression of markers associated with astrocytes should be conducted to conclude on the presence of astrocytes.

As seen in Figure 10 A there was a lower fluorescence output from the cell control placed at the periphery of the plate compared to the cell control surrounded by other wells. Similarly, upon normalizing data to solvent control, a consistent reduction in mitochondrial dehydrogenase activity was observed for the lowest concentration for all chemicals at every time point, although most apparent in the shorter differentiation time points. Visual inspection with phase-contrast microscopy revealed no morphological changes on NSCs although a decreased cell number was observed. Whiteman et al. observed a similar edge effect during

their studies and hypothesized that cell attachment was inhibited due to the rapid heating of the wells along the perimeter of the plate[81]. After framing the plate with PBS, the observed effect on NSCs was eliminated (B). The edge effect affecting the lowest, and possibly the highest, concentration was corrected for by normalizing these concentrations to the mean of the edge wells (G1 and G12) in the solvent control.

5.8 Conclusion

This thesis was part of the project to establish the NSC model at NIPH. By visual inspection with phase-contrast microscopy and HCI and supported by gene expression results of unexposed cells, the culture was found to differentiate as expected. This implies that further results become more credible and contributed to the establishment of a reliable model at NIPH. The cell viability assay was successfully optimized for the NSC model.

We further found that exposure to AA and GA affected the cell viability of NSCs undergoing differentiation with the most apparent effects for immature neurons. Low concentrations of AA and GA, most apparent when combined, resulted in a slight increase in viability, possibly caused by increased proliferation and partly attenuated differentiation. Exposure to higher concentrations of AA and GA resulted in cytotoxicity, probably by both apoptosis and necrosis. When combined, the cytotoxic effects of AA and GA were additive and possibly synergistic, depending on the test concentrations.

AA and GA only slightly affected gene expression of markers related to proliferation, differentiation, neurite outgrowth, and synaptogenesis with no statistically significant alterations. Some trends in expression were observed, most apparent in the combined exposure. There were no long-term alterations in gene expression, however, if the modification was transient it possibly would not have been detected by the current experimental design.

Expression of *GFAP* was not detected over LOD. The presence of astrocytes is an important feature of the NSC model and for its application in DNT testing. However, protein expression may be a more reliable parameter for determining the presence of astrocytes in culture.

Immunocytochemical visualization of protein markers related to neurodevelopment revealed no prominent perturbations in protein expression. Results were qualitative and the alterations would have to be substantial to be visually detectable which makes qualitative evaluations of protein expression less reliable when not supported by quantitative results.

6 Future aspects

Further studies should be performed to assess whether the modifications in viability (mitochondrial activity) upon AA and GA exposure resulted from increased proliferation, decreased cell death, or increased metabolic activity. It would also be interesting to characterize chemical exposure at higher concentrations to see whether cell death resulted from apoptosis or necrosis. Analysis of Hoechst 33342 and propidium iodide together with Ki67 using flow cytometry could differentiate apoptotic from necrotic cells as well as identify and differentiate proliferating NSCs from post-mitotic neurons[82, 83].

The effects of combined AA and GA on cell viability should be repeated with an experimental design more suitable for the determination of the additive effects and potentially interactions (synergism) observed in this study. The individual concentrations of AA and GA in the combination should be equivalent to the concentrations of AA and GA when separately tested. This could provide a better basis for further conclusions regarding the additive, or possibly synergistic or even antagonistic, effect of the combined mixture.

More extensive analysis of gene expression related to neurodevelopment should be included in additional studies. Previous studies have found that micromolar concentrations of AA, relatable to the concentrations used in this thesis, altered expression and activation of neural cell adhesion molecule (NCAM), cAMP response element-binding protein (CREB), and brain-derived neurotrophic factor (BDNF), all important in neurodevelopment[9]. Further studies could include an assessment of a possible altered gene expression in these proteins and signaling pathways. I would also repeat the analysis of the genes used in this thesis in an attempt to reduce the variance and increase the statistical power for å better assessment of the possible human relevance of the results. This should include an experimental design allowing for the investigation of gene expression shortly after exposure as well as long-term effects.

Astrocytic presence in the culture is an important feature of the NSC model and should be investigated further by incorporating alternative genes as well as protein markers related to astrocytes. Immunocytochemical staining with GFAP would be a natural step forward in addition to possibly including other proteins or genes associated with astrocytes e.g. *EAAT2*[84].

Immunocytochemical experiments, complete with quantitative data, could be considered. Quantitative investigations of protein expression would give a more complete image of the possible effect exposure to AA and GA had on neurodevelopment, differentiation, and synaptogenesis. I would also suggest the inclusion of VGlut1 as a marker for glutamatergic neurons[3]. Further immunocytochemical analysis of protein expression, as well as gene expression, related to additional subpopulations of neurons could contribute to further define the regional specificity associated with this NSC model.

Further development of the model could include the establishment of a microelectrode assay (MEA) for measurements of the electric activity of the neurons, including analysis of spike rate, burst count and network burst count to determine the effect of chemical exposure on the number of functional neurons and networks as well as their ability to communicate with each other (i.e. functional parameters).

An *in vitro* system, regardless of how sophisticated, is a great simplification of the complex human brain[85]. To contribute to the determination of *in vitro* to *in vivo* extrapolation of this NSC model future studies should determine the internal concentrations of AA and GA inside the cells upon exposure. By determining the relationship between added exposure in medium and internal cell uptake one could possibly relate DNT effects to fetal brain exposure. By altering the doses used in the *in vitro* NSC model to reflect genuine fetal exposure, possible DNT effects inflicted by chemical exposure could be determined more accurately to improve human risk assessment. Effects on the cellular level should be investigated concurrently with epidemiological studies on cognitive effects in children exposed to AA during pregnancy.

7 REFERENCE LIST

1. Hessel EVS, Staal YCM, Piersma AH. Design and validation of an ontology-driven animal-free testing strategy for developmental neurotoxicity testing. Toxicol Appl Pharmacol. 2018;354:136-52.
2. Silbereis JC, Pochareddy S, Zhu Y, Li M, Sestan N. The Cellular and Molecular Landscapes of the Developing Human Central Nervous System. Neuron. 2016;89(2):248-68.
3. Pistollato F, Canovas-Jorda D, Zagoura D, Price A. Protocol for the Differentiation of Human Induced Pluripotent Stem Cells into Mixed Cultures of Neurons and Glia for Neurotoxicity Testing. J Vis Exp. 2017(124).
4. Erkekoglu P, Baydar T. Acrylamide neurotoxicity. Nutritional Neuroscience. 2013;17(2):49-57.
5. Ghanayem BI, McDaniel LP, Churchwell MI, Twaddle NC, Snyder R, Fennell TR, et al. Role of CYP2E1 in the epoxidation of acrylamide to glycidamide and formation of DNA and hemoglobin adducts. Toxicological Sciences. 2005;88(2):311-8.
6. Hagmar L, Tornqvist M, Nordander C, Rosen I, Bruze M, Kautiainen A, et al. Health effects of occupational exposure to acrylamide using hemoglobin adducts as biomarkers of internal dose. Scand J Work Environ Health. 2001;27(4):219-26.
7. ACS. Acrylamide and Cancer risk. American Cancer Society; 2019.
8. ESFA. Scientific Opinion on acrylamide in food. EFSA Journal. 2015;13(6).
9. Lindeman B, Johansson Y, Andreassen M, Husoy T, Dirven H, Hofer T, et al. Does the food processing contaminant acrylamide cause developmental neurotoxicity? A review and identification of knowledge gaps. Reprod Toxicol. 2021;101:93-114.
10. Garland TO, Patterson MWH. Six Cases of Acrylamide Poisoning. British Medical Journal. 1967;4:134-8.
11. Keeson CM, Lawson DH, Baird AW. Acrylamide Poisoning. Postgraduate Medical Journal. 1977;53:16-7.
12. Jonathan E. Myers, Macurn I. Acrylamide Neuropathy in a South African Factory: An Epidemiologic Investigation. American Journal of Industrial Medicine 1991;19:487-93.
13. Kjuus H, Goffeng LO, Heier MS, Sjoholm H, Ovrebo S, Skaug V, et al. Effects on the peripheral nervous system of tunnel workers exposed to acrylamide and N-methylolacrylamide. Scand J Work Environ Health. 2004;30(1):21-9.
14. Törnqvist M. Acrylamide in Food: The Discovery and Its Implications. A Historicala Perspective [internet]. USA: Springer Science and Business Media; 2004 [cited 01.12.20].
15. Mottram DS, Wedzicha BL, Donson AT. Acrylamide is formed in the Maillard reaction. Nature. 2002;419:448.
16. JECFA. Summary and conclusions of the sixty-fourth meeting of the Joint FAO/WHO Expert Committee on Food Additives Geneva: JECFA; 2005. Report No: 64.
17. Svensson K, Abramsson L, Becker W, Glynn A, Hellenäs KE, Lind Y, et al. Dietary intake of acrylamide in Sweden. Food and Chemical Toxicology. 2003;41(11):1581-6.
18. VKM. Risk assessment of dietary expsure to acrylamide in the Norwegian population Oslo: Norwegian Scientific Committee for Food Safety. 2015.
19. Duarte-Salles T, von Stedingk H, Granum B, Gutzkow KB, Rydberg P, Tornqvist M, et al. Dietary Acrylamide Intake during Pregnancy and Fetal Growth-Results from the Norwegian Mother and Child Cohort Study (MoBa). Environ Health Persp. 2013;121(3):374-9.
20. WHO. Environmental Health Criteria 49 [Internet]. Geneva: WHO; 1985 [cited 03.12.20].

21. Stiles J, Jernigan TL. The basics of brain development. Neuropsychol Rev. 2010;20(4):327-48.
22. Rodier PM. Developing Brain as a Target of Toxicity. Environmental Health Perspectives. 1995;103(6):73-6.
23. Munno DW, Syed NI. Synaptogenesis in the CNS: An Odyssey from Wiring Together to Firing Together. The Journal of Physiology. 2003;552(1):1-11.
24. Webb SJ, Monk CS, Nelson CA. Mechanisms of postnatal neurobiological development: implications for human development. Dev Neuropsychol. 2001;19(2):147-71.
25. Lee S, Park HR, Lee JY, Cho JH, Song HM, Kim AH, et al. Learning, memory deficits, and impaired neuronal maturation attributed to acrylamide. J Toxicol Environ Health A. 2018;81(9):254-65.
26. Attoff K, Kertika D, Lundqvist J, Oredsson S, Forsby A. Acrylamide affects proliferation and differentiation of the neural progenitor cell line C17.2 and the neuroblastoma cell line SH-SY5Y. Toxicol In Vitro. 2016;35:100-11.
27. Attoff K, Johansson Y, Cediel-Ulloa A, Lundqvist J, Gupta R, Caiment F, et al. Acrylamide alters CREB and retinoic acid signalling pathways during differentiation of the human neuroblastoma SH-SY5Y cell line. Sci Rep. 2020;10(1):16714.
28. Chen JH, Chou CC. Acrylamide inhibits cellular differentiation of human neuroblastoma and glioblastoma cells. Food Chem Toxicol. 2015;82:27-35.
29. Park HR, Kim MS, Kim SJ, Park M, Kong KH, Kim HS, et al. Acrylamide induces cell death in neural progenitor cells and impairs hippocampal neurogenesis. Toxicol Lett. 2010;193(1):86-93.
30. Pedersen M, von Stedingk H, Botsivali M, Agramunt S, Alexander J, Brunborg G, et al. Birth weight, head circumference, and prenatal exposure to acrylamide from maternal diet: the European prospective mother-child study (NewGeneris). Environ Health Perspect. 2012;120(12):1739-45.
31. Miller SL, Huppi PS, Mallard C. The consequences of fetal growth restriction on brain structure and neurodevelopmental outcome. J Physiol. 2016;594(4):807-23.
32. Alberts B, Johnson A, Lewis J, Morgan D, Raff M, Roberts K, et al. Molecular Biology of The Cell. 6th ed. USA: Garland Science; 2015.
33. Middeldorp J, Hol EM. GFAP in health and disease. Prog Neurobiol. 2011;93(3):421-43.
34. Bernal A, Arranz L. Nestin-expressing progenitor cells: function, identity and therapeutic implications. Cell Mol Life Sci. 2018;75(12):2177-95.
35. Pistollato F, Louisse J, Scelfo B, Mennecozzi M, Accordi B, Basso G, et al. Development of a pluripotent stem cell derived neuronal model to identify chemically induced pathway perturbations in relation to neurotoxicity: effects of CREB pathway inhibition. Toxicol Appl Pharmacol. 2014;280(2):378-88.
36. Pistollato F, Canovas-Jorda D, Zagoura D, Bal-Price A. Nrf2 pathway activation upon rotenone treatment in human iPSC-derived neural stem cells undergoing differentiation towards neurons and astrocytes. Neurochem Int. 2017;108:457-71.
37. Dehmelt L, Halpain S. the MAP2/Tau family of microtubule-associated proteins. Genome Biology 2004;6(1).
38. Sensenbrenner M, Lucas M, Deloulme J-C. Expression of two neuronal markers, growth-associated protein 43 and neuron-specific enolase, in rat glial cells. Journal of Molecular Medicine. 1997;75(9):653-63.
39. Iovino L, Tremblay ME, Civiero L. Glutamate-induced excitotoxicity in Parkinson's disease: The role of glial cells. J Pharmacol Sci. 2020;144(3):151-64.

40. Lee SE, Lee Y, Lee GH. The regulation of glutamic acid decarboxylases in GABA neurotransmission in the brain. Arch Pharm Res. 2019;42(12):1031-9.
41. Marin O. Interneuron dysfunction in psychiatric disorders. Nat Rev Neurosci. 2012;13(2):107-20.
42. Chinta SJ, Andersen JK. Dopaminergic neurons. Int J Biochem Cell Biol. 2005;37(5):942-6.
43. Howes OD, McCutcheon R, Owen MJ, Murray RM. The Role of Genes, Stress, and Dopamine in the Development of Schizophrenia. Biol Psychiatry. 2017;81(1):9-20.
44. Zheng S, Gray EE, Chawla G, Porse BT, O'Dell TJ, Black DL. PSD-95 is post-transcriptionally repressed during early neural development by PTBP1 and PTBP2. Nat Neurosci. 2012;15(3):381-8, S1.
45. Coley AA, Gao WJ. PSD95: A synaptic protein implicated in schizophrenia or autism? Prog Neuropsychopharmacol Biol Psychiatry. 2018;82:187-94.
46. Kokotos AC, Harper CB, Marland JRK, Smillie KJ, Cousin MA, Gordon SL. Synaptophysin sustains presynaptic performance by preserving vesicular synaptobrevin-II levels. J Neurochem. 2019;151(1):28-37.
47. Kirk RGW. Recovering The Principles of Humane Experimental Technique: The 3Rs and the Human Essence of Animal Research. Sci Technol Human Values. 2018;43(4):622-48.
48. Forskrift om bruk av dyr i forsøk. Oslo: Lovdata.no; 2015, reviced 2017 [internet]
49. Knudsen LE, Smith A, Tornqvist E, Forsby A, Tahti H. Nordic symposium on "toxicology and pharmacology without animal experiments-Will it be possible in the next 10 years?". Basic Clin Pharmacol Toxicol. 2019;124(5):560-7.
50. Norecopa. Norecopa - The frist 10 years. 2017.
51. Bal-Price A, Pistollato F, Sachana M, Bopp SK, Munn S, Worth A. Strategies to improve the regulatory assessment of developmental neurotoxicity (DNT) using in vitro methods. Toxicol Appl Pharmacol. 2018;354:7-18.
52. Price A, Pristollato F, Munn S, Bopp S, Worth A. Stratergic aims for improving the regulatory assessment of Developmental Nurotoxicity (DNT) using non-animal methods. European Commission Joint Research Centre; 2018.
53. Pistollato F, Bremer-Hoffmann S, Healy L, Young L, Stacey G. Standardization of pluripotent stem cell cultures for toxicity testing. Expert Opin Drug Metab Toxicol. 2012;8(2):239-57.
54. Lov om humanmedisinsk bruk av bioteknologi m.m. (bioteknologiloven). Oslo: Lovdata.no; 2003, reviced 2020 [internet]
55. Hølen JC, Bruudgaard D. Forskning på cellelinjer. Tidsskriftet for Den norske Legeforeningen 2012;132(2476).
56. Spinu N, Bal-Price A, Cronin MTD, Enoch SJ, Madden JC, Worth AP. Development and analysis of an adverse outcome pathway network for human neurotoxicity. Arch Toxicol. 2019;93(10):2759-72.
57. Pistollato F, de Gyves EM, Carpi D, Bopp SK, Nunes C, Worth A, et al. Assessment of developmental neurotoxicity induced by chemical mixtures using an adverse outcome pathway concept. Environ Health. 2020;19(1):23.
58. Di Consiglio E, Pistollato F, Mendoza-De Gyves E, Bal-Price A, Testai E. Integrating biokinetics and in vitro studies to evaluate developmental neurotoxicity induced by chlorpyrifos in human iPSC-derived neural stem cells undergoing differentiation towards neuronal and glial cells. Reprod Toxicol. 2020;98:174-88.
59. Bal-Price A, Lein PJ, Keil KP, Sethi S, Shafer T, Barenys M, et al. Developing and applying the adverse outcome pathway concept for understanding and predicting neurotoxicity. Neurotoxicology. 2017;59:240-55.

60. Bal-Price A, Meek MEB. Adverse outcome pathways: Application to enhance mechanistic understanding of neurotoxicity. Pharmacol Ther. 2017;179:84-95.
61. Vinken M. The adverse outcome pathway concept: a pragmatic tool in toxicology. Toxicology. 2013;312:158-65.
62. Coriell Institue for Medical Research. NIA Aging Cell Pepository at Coriell Institute for Medical Research. USA: Coriell Institue for Medical Research; [cited 15.12.20].
63. Davidsen N, Lauvas AJ, Myhre O, Ropstad E, Carpi D, Gyves EM, et al. Exposure to human relevant mixtures of halogenated persistent organic pollutants (POPs) alters neurodevelopmental processes in human neural stem cells undergoing differentiation. Reprod Toxicol. 2020;100:17-34.
64. ThermoFisher. Alamar Blue™ Cell Viability Reagent. 2019.
65. Promega. CellTiter-Blue Cell Viability Assay. 2016.
66. Munshi S, Twining RC, Dahl R. Alamar blue reagent interacts with cell-culture media giving different fluorescence over time: Potential for false positives. Journal of Pharmacological and Toxicological Methods. 2014;70(2):195-8.
67. Watson DE, Li B. TaqMan applications in genetic and molecular toxicology. Int J Toxicol. 2005;24(3):139-45.
68. QiaGen. RNeasy Mini Handbook [Internet]. Germany QiaGen; 2019 [cited 10.03.21].
69. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55(4):611-22.
70. St Laurent G, Shtokalo D, Tackett MR, Yang Z, Vyatkin Y, Milos PM, et al. On the importance of small changes in RNA expression. Methods. 2013;63(1):18-24.
71. Wu YK, Tu YK, Yu J, Cheng NC. The Influence of Cell Culture Density on the Cytotoxicity of Adipose-Derived Stem Cells Induced by L-Ascorbic Acid-2-Phosphate. Sci Rep. 2020;10(1):104.
72. Courchesne E, Mouton PR, Calhoun ME, Ahrens-Barbeau C, Hallet MJ, Barnes CC, et al. Neuron Number and Size in Prefrontal Cortex of Childern with Autism. The Journal of the American Medical Association. 2011;306(18):2001-10.
73. Wei H, Alberts I, Li X. The apoptotic perspective of autism. Int J Dev Neurosci. 2014;36:13-8.
74. Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory [Internet]. Society for the Advancement of Adverse Outcome Pathways (SAAOP); 2015 [updated 07.02.20; cited 05.05.21]. Available from: https://aopwiki.org/aops/17#aop_title.
75. Kole AJ, Annis RP, Deshmukh M. Mature neurons: equipped for survival. Cell Death Dis. 2013;4:e689.
76. Krewski D, Acosta D, Jr., Andersen M, Anderson H, Bailar JC 3rd, Boekelheide K, et al. Toxicity testing in the 21st century: a vision and a strategy. J Toxicol Environ Health B Crit Rev. 2010;13(2-4):51-138.
77. Bu Q, Huang Y, Li M, Dai Y, Fang X, Chen K, et al. Acrylamide exposure represses neuronal differentiation, induces cell apoptosis and promotes tau hyperphosphorylation in hESC-derived 3D cerebral organoids. Food Chem Toxicol. 2020;144:111643.
78. Booth HDE, Hirst WD, Wade-Martins R. The Role of Astrocyte Dysfunction in Parkinson's Disease Pathogenesis. Trends Neurosci. 2017;40(6):358-70.
79. Windrem MS, Osipovitch M, Liu Z, Bates J, Chandler-Militello D, Zou L, et al. Human iPSC Glial Mouse Chimeras Reveal Glial Contributions to Schizophrenia. Cell Stem Cell. 2017;21(2):195-208 e6.

80. Watkins CC, Sawa A, Pomper MG. Glia and immune cell signaling in bipolar disorder: insights from neuropharmacology and molecular imaging to clinical application. Transl Psychiatry. 2014;4:e350.
81. Whiteman MC, Bogardus L, Giacone DG, Rubinstein LJ, Antonello JM, Sun D, et al. Virus Reduction Neutralization Test: A Single-Cell Imaging High-Throughput Virus Neutralization Assay for Dengue. The American Journal of Tropical Medicine and Hygiene. 2018;99(6):1430-9.
82. Kim KH, Sederstrom JM. Assaying Cell Cycle Status Using Flow Cytometry. Curr Protoc Mol Biol. 2015;111:28 6 1- 6 11.
83. Ormerod MG, Sun XM, Brown D, Snowden RT, Cohen GM. Quantification of apoptosis and necrosis by flow cytometry. Acta Oncol. 1993;32(4):417-24.
84. Kim K, Lee SG, Kegelman TP, Su ZZ, Das SK, Dash R, et al. Role of excitatory amino acid transporter-2 (EAAT2) and glutamate in neurodegeneration: opportunities for developing novel therapeutics. J Cell Physiol. 2011;226(10):2484-93.
85. Hartung T. Perspectives on In Vitro to In Vivo Extrapolations. Appl In Vitro Toxicol. 2018;4(4):305-16.

8 Appendix 1: Protocol and information on cell maintenance

Coating of labware

Labware are coated with Matrigel® Basement Membrane Matrix (BMM) for flasks or BMM reduced growth factor (rgf) for plates. BMM and BMM rgf are stored in -20 ºC pending use in aliquot size of 200 µl and 56 µl, respectively. BMM is a soluble membrane consisting of extracellular matrix protein extracted from Enhelbreth-Holm-Swarm mouse sarcoma. The Matrigel's major component is laminin but also contains other proteins and growth factors like collagen IV, heparan sulfate proteoglycan, entracin/nidogen, TGF-beta, EGF, insulin-like GF, FGF, tissue plasminogen activator and other growth factors that occur naturally in the sarcoma. The Matrigel is used for effective attachment of the neurons in culture.

Plates were initially coated from the producer with poly-D-lysine to enhance cell binding to the plate surface. Poly-D-lysine possesses a net positive charge when coated on polystyrene surfaces which enhances cell attachment, growth and differentiation for some cell types. For plates we used Matrigel BMM rgf for additional coating which is purified and characterized to a greater extent than the Matrigel used in flasks.

- Measure out 20 ml DMEM/F12 medium per aliquot of Matrigel®.
- Thaw Matrigel® aliquot by transferring 1 ml DMEM/F12 medium and pipetting slowly.
- Transfer thawed Matrigel® to the remaining DMEM/F12 medium and mix gently before transferring to labware.
- Incubate labware for 1 h at 37 ºC
- Incubate labware for 30 min or till reaching room temperature.

Thawing of cryopreserved cells

- Coat labware according to protocol.
- Prepare NI medium according to protocol.
- Thaw frozen NSC by gentle swirling in water bath (37 ºC) until all visible ice has melted.
- Transfer 1 ml dropwise onto cells and mix by pipetting up and down once.
- Transfer the cells to 5 ml NI medium and mix gently up and down twice.
- Centrifuge the tube at 130 g for 4.5-5 min to remove DMSO remaining from cryopreservation medium.

- Aspirate the supernatant and resuspend the pellet dropwise in 2 ml NI medium
- Add further 4 ml and mix gently by pipetting up and down until cells are completely dissolved (no visible aggregates).
- Assess cell viability by mixing cell suspension 1:1 trypan blue and using an automated cell counter.
- Plate labware in desired cell density, see Table 6.

Table 6. Seeding volume and cell density ideal for post thawing of cryopreserved cells.

Flask size	Seeding volume	Ideal cell number
T25	7ml	$0{,}53x10^6$
T75	20ml	$1{,}6x10^6$

Culture medium

Proliferation medium

NI Complete (NI medium) is made from NI Incomplete consisting of Dulbecco‘s Modified Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with B27 supplement without vitamin A (1x), Bottenstein‘s N2-formulation (N2) (1x), Penicillin-Streptomycin (1,66x), Non-essential amino acid (1x), and Heparin (1mg/ml). NI Complete contains additional factors that stimulate proliferation and suppress differentiation like basic Fibroblast Growth Factor (bFGF) (10ng/ml), Epidermal Growth Factor (EGF) (10ng/ml) and Brain-derived neurotropic factor (BDNF) (2,5ng/ml). bFGF uses heparin as a cofactor and is found to inhibit differentiation and stimulates self-renewal in NSC. EGF is a growth factor used by many cells and is shown to increase proliferation and survival in NSC. BDNF is an important neuronal growth factor involved in neurite outgrowth, synaptogenesis, synapse maturation and stabilization and is added to NI medium in small amounts to aid NSC in survival and is necessary for the cells to be representative for hippocampal and/or cortical neurons in the human body.

Differentiation medium

ND Complete (ND medium) is made from ND Incomplete consisting of Neurobasal Medium supplemented with B27 supplement (1x), Bottenstein‘s N2-formulation (N2) (1x), Penicillin-Streptomycin (1,66x) and L-Glutamine (2nM). ND Complete contains additional factors that stimulates differentiation and survival of NSC like BDNF (2,5ng/ml), Glial Cell Line-derived Neurotropic Factor (GDNF) (1ng/ml) and Laminin (1µg/ml). Laminin support growth and

differentiation of NSC and is the only animal derived product in this thesis. Animal derived laminin is used instead of the human alternative due to the lower cost.

Medium Change

- Preheat Incomplete medium to 37 ºC.
- Prepare the amount of Complete medium needed according to Table 7.
- Aspirate old medium
- For flasks: Tilt flask on the side and add medium gently.
- For plates: Place pipette on side of the well and add medium gently while rotating pipette to avoid mechanic stress.

Table 7. Medium preparation.

Medium	Supplement	Desired Concentration	Growth Factor	Desired Concentration	Dilution
NI	B27 – vit A	1x	BDNF	2,5 ng/ml	1:10.000
	PenStrep	1,66x	EGF	10 ng/ml	1:1000
	N2	1x	bFGF	10 ng/ml	1:1000
	NEAA	1x			
	Heparin	1 mg/ml			
ND	B27	1x	BDNF	2,5 ng/ml	1:10.000
	PenStrep	1,66x	GDNF	1 ng/ml	1:10.000
	L-Glutamine	2mM	Laminin	1 µl/ml	1:1000
	N2	1x			

Passaging of cells

- Aspirate old cell medium from flask.
- Transfer 0,05% Trypsin to cell flask according to Table 8 and incubate at 37 ºC for 2 min.
- Transfer the dissolved cell suspension to a Falcon tube.
- Wash flask with DTI (for volume see Table 8) and transfer the DTI to cell suspension and mix gently.
- Centrifuge suspension for 4.5 min at 130 g.
- Aspirate supernatant tap tube to gently disperse cell pellet and resuspend in NI medium dropwise (Table 8). Mix by pipetting up and down leaving no aggregates.
- Count cell with automated cell counter mixing cell suspension 1:1 with trypan blue.

- Calculate cells and medium needed to make cell suspension and plate labware needed, see Table 9 for seeding volumes.

Table 8. Volumes based on flask size during splitting of cells.

	T25	T75	T150
Transferred diluted Trypsin-EDTA to flask	2 ml	6 ml	12 ml
DTI transferred to flask	2 ml	6 ml	12 ml
NI medium to resuspend pellet	5 ml	10 ml	20 ml

Table 9. Seeding volume, cell density and medium change volume ideal for post passaged cells.

Flask size	Seeding volume	Ideal cell number	Medium Change Volume
T25	7 ml	$0{,}33x10^6$	7-10 ml
T75	20 ml	$0{,}8\text{-}1x10^6$	15-20 ml
T150	40 ml	$2x10^6$	35-40 ml
Plate size			
96-well	135-200µl/well	7000	135-200µl/well
24-well	0,5-1ml/well	24.000	0,5-1ml/well

9 Appendix 2: Products and producers

Reagent name	Producer	Catalogue number	Area of use
Acrylamide	Merck	A3553	Exposure
Alamar Blue™ Cell Viability Reagent	Invitrogen by Thermo Fisher	DAL1025	Alamar Blue™ optimization and Cytotoxicity
B27 Supplement	Thermo Fisher	17504001	Making ND Incomplete medium
B27 Supplement without vitamin A	Thermo Fisher	12587001	Making NI Incomplete medium
BDNF	Thermo Fisher	PHC7074	Making NI and ND Complete medium
bFGF	Thermo Fisher	13256-029	Making NI Complete
BioCoat™ Poly-D-Lysine 24-well Plate	Ibidi	82406	Immunocytochemistry
BioCoat™ Poly-D-Lysine 96-well Plate clear flat bottom	Corning	354461	Alamar Blue™ optimization and Cytotoxicity
Brand® Cryogenic tube	Sigma-Aldrich	BR114840-1000EA	Cryopreservation of cells
BSA 35%	Sigma -Aldrich	A7979-50ML	Immunocytochemistry
CryoStor Cell Cryopreservation medium	Sigma-Aldrich	C2874-100ML	Cryopreservation of cells
DAPI	Thermo Fisher	62248	Immunocytochemistry
Defined Trypsin Inhibitor (DTI)	Thermo Fisher	R007100	Splitting of cells
Destilled water	Locally produced, NIPH		
DMEM/F12 GlutaMax	Thermo Fisher	31331028	Making NI medium and Coating
EGF	Thermo Fisher	PHG6045	Making NI Complete medium
Ethanol 97%	Antibac	600068	
Falcon™ Tubes 15ml and 50 ml	Falcon	339650 339652	Medium preparation
Flacon™ Tissue Culture Treated Flasks T25/T75/T150	Flacon	353108 353136 355001	Cultivation of NSC
Formaldehyde 36%	Sigma-Aldrich	47608-250ML-F	Fixation

GABA primary antibody	Sigma Aldrich	A0310	Immunocytochemistry
GDNF	Thermo Fisher	PHC7045	Making ND Complete medium
Glycidamide	Merck	04704	Exposure
Goat Anti-Chicken IgY H&L (DyLight 488) preabsorbed	Abcam	Ab96951	Immunocytochemistry
Goat Anti-Mouse IgG H&L (DyLight 550) preabsorbed	Abcam	Ab96880	Immunocytochemistry
Goat Anti-Rabbit IgG H&L (DyLight 650)	Abcam	Ab96902	Immunocytochemistry
Heparin	Sigma-Aldrich	H3149-100KU	Making NI Incomplete medium
Ki67 primary antibody	Merck	MAB4190	Immunocytochemistry
Laminin	Sigma-Aldrich	L2020-1MG	Making ND Complete medium
L-Glutamine	Thermo Fisher	25030024	Making ND Incomplete medium
MAP2 primary antibody	Abcam	Ab5392	Immunocytochemistry
Matrigel Basement Membrane Matrix	Corning	354234	Coating for flasks
Matrigel Basement Membrane Matrix Reduced Growth Factor	Corning	354230	Coating for plates
Medium bottle 125 ml, 250 ml, and 500 ml	Fisherbrand™		Medium preparation
N2 Supplement	Thermo Fisher	25030024	Making NI and ND Incomplete medium
Nestin primary antibody	Abcam	N5413-100UG	Immunocytochemistry
Neural Induction Medium	Thermo Fisher	A1647801	Making NI medium
Neurobasal Medium	Thermo Fisher	21103049	Making ND medium
Non-essential amino acid	Thermo Fisher	11140-035	Making NI Incomplete medium
PBS with Ca^{2+} and Mg^{2+} (1x)	Thermo Fisher	14040133	Fixation and Immunocytochemistry
PBS Without Ca^{2+} and Mg^{2+}	Thermo Fisher	14190250	Splitting of cells and during experiments to fill edge wells

PBS without Ca^{2+} and Mg^{2+} (1x)	Thermo Fisher	14190250	Fixation and Immunocytochemistry
Penicillin/Streptomycin	Thermo Fisher	15140-122	Making NI and ND Incomplete medium
PSD95 primary antibody	Abcam	Ab13552	Immunocytochemistry
QiaShredder (250)	Qiagen	79656	RNA preparation
RNase away	Thermo Fisher	7005-11	RNA preparation
RNasy Mini kit	Qiagen	74104	RNA preparation
RNeasy Micro kit	Qiagen	74004	RNA preparation
Synaptophysin primary antibody	Abcam	AB14692	Immunocytochemistry
TaqMan Gene Expression Assay	Thermo Fisher	4331182	Gene Expression analysis
TaqMan Gene Expression Master Mix	Thermo Fisher	4369016	Gene Expression analysis
TH primary antibody	Millipole	AB152	Immunocytochemistry
Triton X-100 solution	Sigma-Aldrich	93442-100ML	Immunocytochemistry
Trypan Blue (0,4%)	Sigma-Aldrich	T8154-100ML	Splitting of cells
Trypsin-EDTA (0,5%)	Thermo Fisher	15400054	Splitting of cells
384-well PCR plate	Bio-Rad	HSP3805	PCR

10 Appendix 3: Preparation of substance stock solutions

Table 10. Preparation of sub-stock solutions from 3 M stock solutions for exposure of NSC. Calculations was done using formula: $C_1*V_1=C_2*V_2$. Sub-stock for the whole experiment was prepared at once to minimize dilution variation within an experiment and kept in -80 ºC between use.

Acrylamide and Glycidamide												
Original stock concentration (S_{orig})			3M									
Sub stock (S_x):	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	S_{12}
Stock added	40µl from S_{orig}	15µl from S_{orig}	4,5µl from S_{orig}	4,5µl from S_2	4,5µl from S_3	4,5µl from S_4	4,5µl from S_5	4,5µl from S_6	4,5µl from S_7	4,5µl from S_8	4,5µl from S_9	4,5µl from S_{10}
H_2O added	0µl	30µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl
Final volume	40µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl
Final Concentration	3M	1M	$3x10^{-1}$M	$1x10^{-1}$M	$3x10^{-2}$M	$1x10^{-2}$M	$3x10^{-3}$M	$1x10^{-3}$M	$3x10^{-4}$M	$1x10^{-4}$M	$3x10^{-5}$M	$1x10^{-5}$M
Combination of Acrylamide and Glycidamide												
Acrylamide						Glycidamide						
Original stock concentration (S_{origAA})			3M			Original stock concentration (S_{origGA})				3M		
Sub stock (S_x):	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	S_{12}
Stock added	30µl from S_{origAA} and S_{origGA}	15µl from S_1	4,5µl from S_1	4,5µl from S_2	4,5µl from S_3	4,5µl from S_4	4,5µl from S_5	4,5µl from S_6	4,5µl from S_7	4,5µl from S_8	4,5µl from S_9	4,5µl from S_{10}
H_2O added	0µl	30µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl
Final volume	60µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl	40,5µl
Final Concentration	3M	1M	$3x10^{-1}$M	$1x10^{-1}$M	$3x10^{-2}$M	$1x10^{-2}$M	$3x10^{-3}$M	$1x10^{-3}$M	$3x10^{-4}$M	$1x10^{-4}$M	$3x10^{-5}$M	$1x10^{-5}$M

11 Appendix 4: Alamar Blue™ cell viability assay

Plate Layout for the cytotoxicity experiment

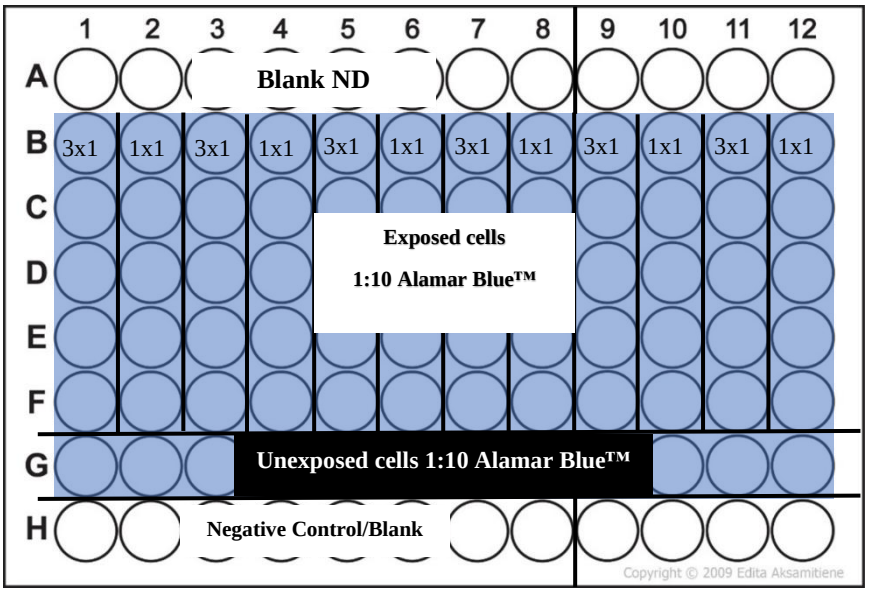


Figure 16. ***Plate layout for cytotoxicity experiment with cell viability measured by Alamar Blue™ assay****. Column 1 B-F contains the highest concentration, $3x10^{-3}$M, followed by $1x10^{-3}$M. Concentrations decrease 10^{-1}M for every other column till column 12 which contain the lowest concentration of $3x10^{-8}$M. Row G contain unexposed cells working as a solvent control. During differentiation Row A and H contain PBS at a corresponding volume. Under Alamar Blue™ assay, Row A wells 1-8 contains a blank with ND medium only corresponding to the total volume. Row B-G contains chemically exposed cells (Row B-F) and a positive control (Row G) added 1:10 dilution of Alamar Blue™ Cell Viability Reagent directly added to ND medium. Row H wells 1-8 function as a negative control containing ND medium and Alamar Blue™ Cell Viability Reagent with the total volume corresponding to other wells. Remaining wells A 9-12 and H9-12 contain PBS at a corresponding volume.*

Plate Layout for the optimization of Alamar Blue™

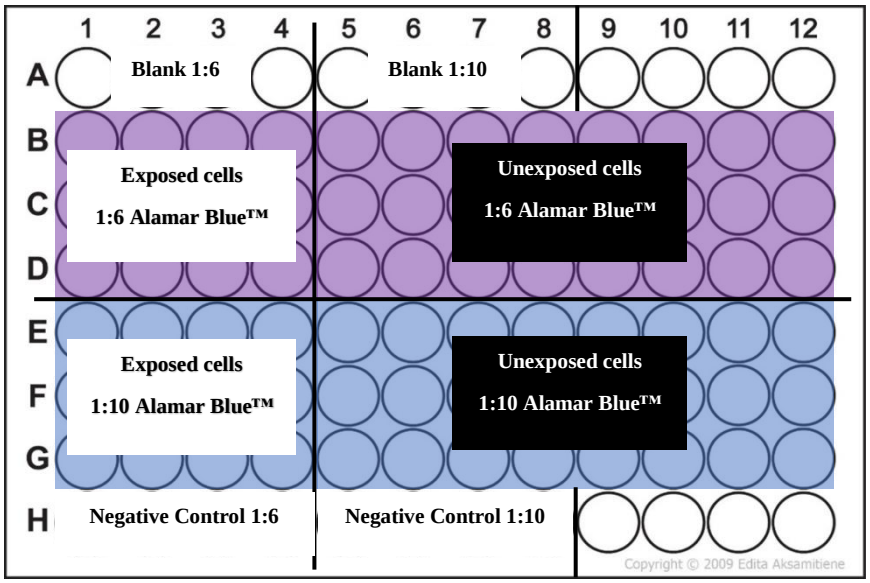


Figure 17. ***Plate layout for optimization experiment.*** *Row A well 1-4 contains a blank corresponding to the volume in wells with cells with 1:6 dilution of Alamar Blue™. Row A well 5-8 contains a blank corresponding to the volume in wells with cells with 1:10 dilution of Alamar Blue™. Row B-G wells 1-4 contains cells exposed to $1x10^{-3}$ M glycidamide in ND medium exposed for 24h. Row B-G wells 5-12 contains unexposed cells in ND medium and function as positive control cells. 1:6 dilution of Alamar Blue™ was added to row B-D wells 1-12. 1:10 dilution of Alamar Blue™ was added to row E-G wells 1-12. Row H function as a negative control with volumes corresponding to the volumes in wells with 1:6 dilution, well 1-4, and 1:10 dilution, well 5-8, of Alamar Blue™.*

Statistical effects of AA and/or GA on NSCs undergoing differentiation when compared to NSCs differentiated for 1 day.

*Table 11. Effects of differentiation time on NSCs exposed to twelve concentrations ranging from $3x10^{-3}$ M to $1x10^{-8}$ M of AA and GA when compared to nearly undifferentiated cells. Non-parametric analysis Steel Method is used for statistical calculations. *p<0,05, **p<0,01, ***p<0,001 compared to DIV1 as nearly undifferentiated control. ns = not significant from DIV1. DIV = days of combined differentiation and exposure.*

	Chemical →	AA			GA			COMBO		
	DIV →	DIV3	DIV14	DIV21	DIV3	DIV14	DIV21	DIV3	DIV14	DIV21
Concentration →	$3x10^{-3}$ M	***	***		***	***		***	***	
	$1x10^{-3}$ M	***	***		***	***		***	***	
	$3x10^{-4}$ M	ns	**		*	***		*	***	
	$1x10^{-4}$ M	*	ns		ns	***		ns	***	
	$3x10^{-5}$ M	ns	*		ns	**		ns	***	
	$1x10^{-5}$ M	*	***	***	ns	ns	**	ns	***	***
	$3x10^{-6}$ M	*	***		ns	ns		ns	***	
	$1x10^{-6}$ M	ns	**	***	ns	ns	*	ns	***	***
	$3x10^{-7}$ M	*	**		ns	ns		ns	***	
	$1x10^{-7}$ M	ns	*	***	ns	ns	ns	ns	***	***
	$3x10^{-8}$ M	ns	ns		**	**		ns	ns	
	$1x10^{-8}$ M	ns	ns		**	***		**	***	