Cell models for evaluation of adult and developmental neurotoxicity
Focus on acrylamide
Kristina Attoff

Abstract
This thesis is aimed at summarizing some of the alternative in vitro methods and models that have been used to study both adult and developmental neurotoxicity (DNT), and also to pinpoint some of the important aspects of using alternative in vitro methods. The aim of the papers included in this thesis was to challenge the hypothesis that neurotoxicity and DNT of chemicals can be studied using robust endpoints for proliferation and neural differentiation, such as neurite outgrowth, mRNA expression and protein expression, in two different cell lines. The aim was also to characterize the two cell lines and identify marker genes important for differentiation and to evaluate if these markers could be used as indicators for DNT. The hypothesis being that any chemical that change the expression of important genes for the developmental process could possibly result in DNT for the cells. The current developmental neurotoxicity testing guidelines, using animal models, are time consuming, expensive, ethically questionable and have relatively low sensitivity. Because of this, there has been a paradigm shift towards developing and using alternative methods capable of testing and screening large number of substances. The next generation of developmental neurotoxicity testing is predicted to consist of both in silico and in vitro testing that have to be used in a combined fashion so that it will generate a more rapid and efficient toxicity testing. The idea is to use a battery of refined endpoint studies that identify the specific toxicity of a compound, discriminate between different neural subpopulations and the different stages of neural differentiation. The use of transcriptomic approaches has been suggested as an example of such an endpoint. In this thesis we have evaluated the human neuroblastoma cell line SH-SY5Y and the murine neural progenitor cell line C17.2 in their ability to detect neurotoxic and developmental neurotoxic compounds. We have evaluated this by using functional endpoints, such as neurite outgrowth, cell membrane potential and phenotype ratios. We have also studied the effect of selected chemicals on the levels of mRNA markers specific for different neural cell populations or for neural differentiation in general. We have performed whole genome gene expression on the two cell lines during differentiation and identified and selected a limited number of genes that have been evaluated for their ability to detect developmental neurotoxicity. Both cell lines showed that they have the capability to identify neurotoxic and developmental neurotoxic compounds and could possibly serve as an addition to the testing battery of neurotoxicity in the future. Some of the focus of this thesis has been directed towards the neurodevelopmental effects of the neurotoxic compound acrylamide. Most people get exposed to acrylamide through food consumption and from environmental pollution. Since acrylamide crosses the placental barrier, it creates a risk for developmental consequences. We found that acrylamide affected both cell proliferation and differentiation in both cell lines. Acrylamide affected both neuronal and the glial phenotypes in the C17.2 cell line. We also revealed that acrylamide attenuated neural differentiation at concentrations that were seven orders of magnitude lower than the estimated plasma concentration of free acrylamide in the fetus. Low concentrations of acrylamide altered the gene expression of several genes involved in the retinoic acid signaling as well as the CREB signaling pathways during retinoic acid driven differentiation in the SH-SY5Y cells. Since sub-micromolar concentrations seem to inhibit the differentiation process in both cell lines, developmental neurotoxicity induced by daily intake of acrylamide is a matter of concern. We found that the C17.2 cell line could function as a good model for detecting acute neurotoxicity by evaluating the cell membrane potential of the cells in combination with gene expression of neural and stress marker genes.
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Sho till hela min familj,
utan er jag ingenting.
List of publications

I. Attoff K., Kertika D., Lundqvist J., Oredsson S. and Forsby A
Acrylamide affects proliferation and differentiation of the neural progenitor cell line C17.2 and the neuroblastoma cell line SH-SY5Y
Toxicology in Vitro, 2016, 35, 100–111

II. Attoff K., Gliga A., Lundqvist J., Norinder U. and Forsby A
Whole genome microarray analysis of neural progenitor C17.2 cells during differentiation and validation of 30 neural mRNA biomarkers for estimation of developmental neurotoxicity
PLoS One, 2017, 12(12), 1-29

III. Attoff K., Gliga A., Johansson Y., Cediel Ulloa A., Lundqvist J and Forsby A
Acrylamide alters CREB and retinoic acid signaling pathways during differentiation of the human neuroblastoma SH-SY5Y cell line
Manuscript

IV. Lundqvist J., Svensson C., Attoff K., and Forsby A
Altered mRNA expression and cell membrane potential in the differentiated C17.2 cell model as indicators of acute neurotoxicity
Applied In Vitro Toxicology, 2016, 3(2), 154-162
Abstract

This thesis is aimed at summarizing some of the alternative in vitro methods and models that have been used to study both adult and developmental neurotoxicity (DNT), and also to pinpoint some of the important aspects of using alternative in vitro methods. The aim of the papers included in this thesis was to challenge the hypothesis that neurotoxicity and DNT of chemicals can be studied using robust endpoints for proliferation and neural differentiation, such as neurite outgrowth, mRNA expression and protein expression, in two different cell lines. The aim was also to characterize the two cell lines and identify marker genes important for differentiation and to evaluate if these markers could be used as indicators for DNT. The hypothesis being that any chemical that change the expression of important genes for the developmental process could possibly result in DNT for the cells. The current developmental neurotoxicity testing guidelines, using animal models, are time consuming, expensive, ethically questionable and have relatively low sensitivity. Because of this, there has been a paradigm shift towards developing and using alternative methods capable of testing and screening large number of substances. The next generation of developmental neurotoxicity testing is predicted to consist of both in silico and in vitro testing that have to be used in a combined fashion so that it will generate a more rapid and efficient toxicity testing. The idea is to use a battery of refined endpoint studies that identify the specific toxicity of a compound, discriminate between different neural subpopulations and the different stages of neural differentiation. The use of transcriptomic approaches has been suggested as an example of such an endpoint. In this thesis we have evaluated the human neuroblastoma cell line SH-SY5Y and the murine neural progenitor cell line C17.2 in their ability to detect neurotoxic and developmental neurotoxic compounds. We have evaluated this by using functional endpoints, such as neurite outgrowth, cell membrane potential and phenotype ratios. We have also studied the effect of selected chemicals on the levels of mRNA markers specific for different neural cell populations or for neural differentiation in general. We have performed whole genome gene expression on the two cell lines during differentiation and identified and selected a limited number of genes that have been evaluated for their ability to detect developmental neurotoxicity. Both cell lines showed that they have the capability to identify neurotoxic and developmental neurotoxic compounds and could possibly serve as an addition to the testing battery of neurotoxicity in the future. Some of the focus of this thesis has been directed towards the neurodevelopmental effects of the neurotoxic compound acrylamide. Most people get exposed to acrylamide through food consumption and from environmental pollution. Since acrylamide crosses the placental barrier, it creates a risk for developmental consequences. We found that acrylamide affected both cell proliferation and differentiation in both cell lines. Acrylamide affected both neuronal and the glial phenotypes in the C17.2 cell line. We also revealed that acrylamide attenuated neural differentiation at concentrations that were seven orders of magnitude lower than the estimated plasma concentration of free acrylamide in the fetus. Low concentrations of acrylamide altered the gene expression of several genes involved in the retinoic acid signaling as well as the CREB signaling pathways during retinoic acid driven differentiation in the SH-SY5Y cells. Since sub-micromolar concentrations seem to inhibit the differentiation process in both cell lines, developmental neurotoxicity induced by daily intake of acrylamide is a matter of concern. We found that the C17.2 cell line could function as a good model for detecting acute neurotoxicity by evaluating the cell membrane potential of the cells in combination with gene expression of neural and stress marker genes.
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Abbreviations

5-HT    serotonin
ACh     acetylcholine
ACR     acrylamide
ADHD    attention deficit hyperactivity disorder
AKT     protein kinase B
BBB     Blood-brain barrier
BDNF    brain-derived neurotrophic factor
BrdUrd  5-bromo-2’-deoxyuridine
cDNA    complimentary DNA
C.elegans  Caenorhabditis elegans
CNS     central nervous system
CGC     cerebellar granule cell
CRABP   cellular retinoic acid-binding protein
CREB    cAMP response element binding protein
CREM    cAMP response element modulator
cRNA    complimentary RNA
Ct      cycle threshold
DA      dopamine
DNA     deoxyribonucleic acid
DNT     developmental neurotoxicity
dUTP    deoxyuridine triphosphate
E       embryonic day
ECH A   European chemicals agency
ECVAM   European center for the validation of alternative methods
ELISA   enzyme-linked immunosorbent assay
EPA     United States environmental protection agency
ER      endoplasmic reticulum
ESC     embryonic stem cell
EURL ECVAM European Union reference laboratory for alternatives to animal testing
GABA    gamma-aminobutyric acid
GFAP    glial fibrillary acidic protein
GW      gestational week
H2O2    hydrogen peroxide
HDAC    histone deacetylase
HRP     horse radish peroxidase
iPSC    induced pluripotent stem cells
NA      noradrenalin
nAChR   nicotinic acetylcholine receptor
NGF     nerve growth factor
NMDA    N-methyl-D-aspartate
NO      nitric oxide
NPC     neural progenitor cell
NRC     national research council
NSC  neural stem cells  
mAchR  muscarinic acetylcholine receptor  
MAM  methylazoxymethanol  
mBDNF  mature brain-derived neurotrophic factor  
MeHg  methylmercury  
MOA  mode of action  
mRNA  messenger RNA  
MTT  3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
OECD  organization for economic co-operation and development  
P75NTR  p75 neurotrophin receptor  
PC12  pheochromocytoma 12  
PCR  polymerase chain reaction  
PI  propidium iodide  
PKA  protein kinase A  
PKC  protein kinase C  
PNS  peripheral nervous system  
QSAR  quantitative structure-activity relationship  
RA  all-trans-retinoic acid  
RAR  nuclear RA receptors  
RARE  RA response element  
REACH  registration, evaluation, authorization and restriction of chemical substances  
RNA  ribonucleic acid  
ROS  reactive oxygen species  
RSK  ribosomal s6 kinase  
RT-qPCR  quantitative reverse transcription polymerase chain reaction  
RXR  retinoid X receptor  
SSC  somatic stem cell  
TrkB  tropomyosin receptor kinase B  
VPA  valproic acid
Introduction

The nervous system
Nervous systems with varying complexities are organ systems found in most multicellular organisms. The vertebrate nervous system is structurally composed of two integrally linked parts: the central nervous system (CNS), consisting of the brain and spinal cord, and the peripheral nervous system (PNS), consisting of motor-, sensory, cranial- and spinal nerves and autonomic ganglia. For a long time, it was generally accepted that the human brain consisted of an average of 100 billion neurons. However, there is no peer-reviewed scientific paper published supporting that number. More recent studies have found that the average human brain consist of approximately 86 billion neurons. These studies have considered the different neuronal densities of different brain regions, e.g. cerebellum contains over 50% of the neurons of the brain but only represents about 10% of the mass. Interestingly enough, this number is similar to the number of neurons in the brain of a baboon. The nervous system is responsible for countless vital functions in the body (such as breathing, swallowing and heartbeat) and any type of damage to its functions can have detrimental effects for the organism.

Development of the nervous system
Towards the end of the second week after conception, the embryo is an oval-shaped structure consisting of two different layers. The upper layer consists of epiblast cells and the lower layer consists of hypoblast cells. After another week, towards the end of the third week after conception, gastrulation occurs which transforms the embryo from a two-layered structure into a three-layered structure. This is an extremely important event since the transformations that occur during gastrulation lay the foundation for the rest of the developmental processes. The upper layer consisting of epiblast cells will give rise to all three primary germ layers (ectoderm, mesoderm and endoderm) and the lower layer consisting of the hypoblast cells give rise to the yolk sac and will later give rise to the fetal component of the placenta. In short, the ectoderm differentiates to form the nervous system, tooth enamel and the epidermis. At least for vertebrates, the ectoderm has three parts: external ectoderm, the neural crest, and neural tube. The neural crest and the neural tube are also described as the neuroectoderm. The neural tube cells will become the CNS cells whilst the neural crest cells will become the peripheral and enteric nervous system. The most anterior part of the neural tube balloons into three primary vesicles: the forebrain (prosencephalon), the midbrain (mesencephalon), and the hindbrain (rhombencephalon).
Figure 1. A timeline over the different processes that occur during the development of the nervous system.
As depicted above, the development of the nervous system is extremely complex. The development of the CNS begins during the third gestational week in humans and continues throughout adolescence, debatably even throughout life.\textsuperscript{7} The development of the nervous system is driven by both gene expression and the environment and consists of a detailed and controlled series of dynamic and adaptive processes including proliferation, migration, differentiation, synaptogenesis, apoptosis and myelination\textsuperscript{10,11} (Figure 1). If one or several of these processes are disrupted, e.g. due to chemical insult, it could result in permanent developmental damages in the offspring.\textsuperscript{12} Since the different developmental processes take place during well-defined time windows, it can make the developing nervous system sensitive to particular substances at specific times, whilst them being totally harmless at other time points.\textsuperscript{10,13} One example of this is during development of cerebellum where Purkinje cells develop earlier than the granule cells. Purkinje cells develop during E13–15 in rats, analogous to GW 5–7 in humans and granule cells develop during postnatal days 4–19 in rats, analogous to GW 24–36 in humans.\textsuperscript{14} Hence, it is not only a question of accessibility of a substance but also at during which specific time the nervous system gets exposed during development.

Not surprisingly, it has been shown that the developing CNS is susceptible to low doses of substances that are harmless to the mature CNS.\textsuperscript{10,15,16} The fetus is somewhat protected by the placenta but some substances can easily pass due to their physicochemical properties and interactions with other molecules and proteins.\textsuperscript{17} Neither the blood-brain barrier (BBB), nor the blood-cerebrospinal fluid barrier is completely formed until approximately 6 months after birth in humans. This gives some substances access to the fetal brain that would normally be excluded in the adult brain.\textsuperscript{17–19} Since the CNS continues to develop from birth until early adulthood,\textsuperscript{20} the brain is vulnerable for an extended period of time, possibly throughout our lifetime. The particular susceptibility of children is further increased due to their fast absorption rates and diminished ability to detoxify many exogenous substances.\textsuperscript{21} Also, the impulsive behavior of children puts them at greater risk for acute accidental exposure to toxic substances.\textsuperscript{22,23}

The adult nervous system

Once developed, the CNS is composed of many different brain regions that in turn consist of an array of different cell types that are responsible for a variety of functions. There are two major cell types in the nervous system, i.e. neurons and the non-neuronal glial cells. In the CNS, glial cells comprise oligodendrocytes, astrocytes, ependymal cells, and microglia. In the PNS, glial cells include Schwann cells and satellite cells. The glial cells actually make up for 33–66\% of the total brain mass, depending on the mammalian species.\textsuperscript{24} The different types of glial cells have varying functions. In short, microglia are phagocytotic, immunocompetent cells that originate from the yolk sac progenitors, which are only present in the brain during its development.\textsuperscript{25} The remaining cell types all originate from the ectodermal tissue. Astrocytes constitute the most represented glial cell type in the brain and has a wide array of functions such as ion homeostasis, maintenance of the BBB, oxygen and nutrient provision and they also have a vital role in synaptic transmission.\textsuperscript{26} In addition, astrocytes have been shown to influence the adverse effects of different toxic substances due to their metabolic capacity.\textsuperscript{27} Oligodendrocytes are sometimes divided into mature oligodendrocytes and NG2-glia, which are precursors of mature oligodendrocytes.\textsuperscript{28} The exact function of the NG2-glia is still mostly unknown in the adult brain but studies in rodents have shown that they form a homeostatic network regulating the number of cells present under physiological conditions.\textsuperscript{29} Mature oligodendrocytes are the myelinating cells of the CNS (represented by the Schwann cells in the PNS). They provide the
neurons with structural and trophic support as well as increasing the speed of conduction. Ependymal cells line the cerebrospinal fluid-filled ventricles in the brain and the central canal of the spinal cord where they produce cerebrospinal fluid. When it comes to neurons, they can be classified by morphology, which neurotransmitter they release or electrophysiological properties. There are four major divisions of classes defined by fundamental connectional input-output relationships: sensory, motor, sensory-motor and interneurons. In the spinal cord, neurons are often classified after their function whilst in the brain, the different neuronal phenotypes tend to be classified after the neurotransmitter they release, e.g. GABAergic, glutamatergic and serotonergic neurons. Adult neural stem cells have taken a central role in research during the last decades. Studies have shown that there is adult neurogenesis taking place in the subgranular zone of the dentate gyrus of the adult mammalian hippocampus (most studies are performed in rodents). In humans, there are studies indicating that there is adult neurogenesis taking place in hippocampus, generating up to 700 new neurons per day. However, lately there have been studies reporting that adult neurogenesis is extremely scarce and mostly takes place in early childhood, giving the explanation to why the few proliferating cells in the adult brain are majorly microglia. From a neurotoxicological point of view, the complexity of the nervous system with its many diverse cell types and functions creates a vast number of events that might lead to adverse effects. If adult neurogenesis is a crucial part of a functioning brain, chemicals interfering with proliferation of stem cells as well as affecting neuronal differentiation might not only be toxic during development, but also throughout life to varying degrees. A lot of the targets of known neurotoxic compounds are different neuronal receptors involved in the transmission of action potentials or neurotransmitters, such as ligand-gated ion channels, voltage-operated ion channels or G-protein coupled receptors.

Toxicological assessment and regulation of chemicals

Toxicology
The term toxicology derives from the Greek words toxicos (poisonous) and logos (word). It refers to the study of all adverse effect in a living biological organism that is produced by a chemical. Those adverse events can be any alteration from the cellular, biochemical or macromolecular functions of the organism. The term toxicology sometimes also includes prevention and amelioration of adverse effects. By definition, toxins are substances that are produced by living organisms whilst substances that are synthesized by man or pollutants found in the environment are called toxicants. Both toxins and toxicants are amongst the most hazardous substances known to mankind, e.g. botulinum toxin, generated by the bacterium Clostridium botulinum, or metal toxicants like mercury or arsenic.

Paracelsus, who was a Swiss physician, alchemist, and astrologer of the German Renaissance, is sometimes called the father of toxicology since he founded the concept of dose response. He said “Sola dosis facit venenum”, which in English translates into “The dose makes the poison”. This occurred during his third defense that was held after his discovery that mercury, although being tremendously toxic, could actually treat syphilis at low doses (Paracelsus, 1493-1541). While this is true for many substances, far from all substances display a dose-dependent response and might be extremely toxic at low levels during chronic exposure.
Regulation of chemicals

There has been an increased regulation of environmental chemicals over the past 60 years. This is partly due to the fact that there is an increasing number of chemicals being used, but also due to our increasing knowledge of what implications these chemicals might have on our environment and on human health. Thousands of chemicals are on the market worldwide and the number is constantly increasing as new chemicals are developed. In the United States, the Toxic Substances Control Act demands that the U.S. Environmental Protection Agency (EPA) has a list of all chemicals that are being processed or manufactured in the country. This list contains around 86,228 chemicals. However, according to a new report published in February 2019, the percentage of chemicals used in commerce and that are produced in what EPA considers as significant amounts is 47% (or 40,655 of the 86,228 chemicals). According to a report published by the United States government accountability office (GAO), around 1,500 new chemicals are introduced each year on the United States market. In Europe, the regulation that regulates chemicals and their safe use is called REACH (Registration, Evaluation, Authorization and Restriction of Chemical substances). REACH was enforced on the 1st of June 2007 and the purpose of REACH is to protect human health and the environment from all risks that come from man-made chemicals. REACH applies to all chemicals including substances we are exposed to in our everyday life such as chemicals from cleaning supplies, furniture, electronics and clothes. This means that this regulation has an impact on most companies in Europe. According to the REACH regulation EC No 1907/2006 of the European Parliament and of the Council of 18 December 2006, chemicals produced or imported to the EU in high volumes (<1,000 tons per year) were to be registered to the European chemicals agency (ECHA) by the year 2010, middle volumes (100-1,000 tons per year) by 2013 and low volumes (1-100 tons per year) by 2018. The chemicals need to be registered and the manufacturer/importer must also provide appropriate safety data to the agency. ECHA has ever since collected the data and provides a public database of the labeling, classification and safety information submitted for all chemicals registered. ECHA also evaluates the information provided by the industries for different chemicals, and if it is insufficient they will require additional information. When writing this, there are currently 24,558 chemicals registered for commerce at ECHA within the EU. The amount of safety information required for a chemical, including different toxicity studies, increases with the amount that is produced per year. The Member States and the European Parliament, together with the scientific committees at ECHA will decide on restrictions for any particular chemical. After a restriction has been made, it is the Competent Authorities in the Member States that are responsible for enforcing the restriction. The goal of this regulation is to raise awareness about the chemicals that are being used so that the most toxic chemicals can be phased out and replaced with safer options.

European center for validation of alternative methods and the 3R principle

REACH provides alternative methods for toxicity testing in accordance with the principles of the 3Rs (reduction, refinement and replacement of animal testing). The concept of the 3Rs was first described by Russell and Burch in 1959 in their book “The Principles of Humane Experimental Technique”. Today this concept is taken into consideration in the legislation regarding animals for experimental use in many countries. In need of additional testing, alternative methods should be considered before using vertebrate animals (article 13 of REACH). Since 1991, the European center for the validation of alternative methods (ECVAM) has been the organization within the EU that are coordinating the validation and acceptance of alternative in vitro testing models to be used for regulatory risk assessment. Since 2011, the tasks assigned to ECVAM are executed by the European Union reference laboratory for alternatives to animal testing (EURL ECVAM). The laboratory is hosted by the Joint Research
Center, Institute for Health and Consumer Protection. According to the National Research Council of the US National Academies of Science (NRC), *in vitro* methods are the best and most efficient way to study the toxicity of large quantities of chemicals, including developmental neurotoxicity (DNT) testing. The demand on organizations like REACH to test a high number of chemicals in a short amount of time calls for the development of rapid, efficient, reliable and cheap alternative methods for testing of chemicals.

**Models to define acute toxicity – OECD test guidelines**

Historically speaking, acute oral toxicity has been determined mostly by the lethal dose 50% (LD50) that was described in the Organization for Economic Co-operation and Development (OECD) test guideline 401. This particular test guideline has been retracted since 2002 but in short term, the LD50 value is defined as the single dose that kills 50% of the animals in a group. The value is estimated from dose-response curves ranging from subtoxic doses to extremely high doses that generate 100% lethality. The use of the OECD test guideline 401 was debated for years due to the vast number of animals being used, the suffering of the animals and also for its relevance. The test guidelines that exist today to study acute oral toxicity are modified versions of the 401 test guideline and are considered to be more humane, i.e. OECD test guidelines 420, 423 and 425. One example to reduce animal suffering is described in test guideline 420, where fixed doses are being used. The idea is that only moderately toxic doses should be used and doses that are expected to be lethal should be avoided. A recent report from the ECHA states that there is great progress regarding alternatives to animal testing on the lower tier properties of substances, e.g. for skin corrosion/irritation, serious eye damage/eye irritation and skin sensitization. Many companies already use *in vitro* methods on a regular basis to study such parameters. However, the report states that for more complex endpoints such as reproductive, developmental or repeated dose toxicity, alternatives to animal testing are insufficient and not yet acceptable. It should be mentioned that *in vitro* studies might be used in combination with more complex models or in read-across or weight of evidence based evaluations.

**Acute and chronic toxicity – definitions and OECD test guidelines**

The definitions of acute, sub-acute and chronic toxicity can differ somewhat between different countries and legislative organs. The Globally Harmonized System (GHS) defines acute toxicity as all adverse effects that occur after oral (OECD test guidelines 420, 423 and 425) or dermal administration (OECD test guideline 402) of a single or multiple doses of a substance within 24 hours, or for inhalation studies an exposure of 4 hours (OECD test guideline 403). The GHS defines subacute, subchronic and chronic toxicity as specific target organ/systemic toxicity arising from a repeated exposure. The exposure routes can be the same as for acute toxicity, i.e. oral (OECD test guidelines 407 and 408), dermal (OECD test guidelines 410 and 411) and by inhalation (OECD test guidelines 412 and 413). In these studies, repeated dose toxicity in rat is studied for 28 days (subacute exposure) or for 90 days (subchronic exposure) or up to a year (chronic exposure). The endpoints for these studies are primarily effects on various organ systems and the goal is to establish a no effect dose level. There is usually an evaluation of both clinical observations, blood analysis and histopathological examinations of tissue and organ samples.
Neurotoxicity – definition and OECD test guidelines
If a chemical shows signs of potential neurotoxicity, the chemical can be further evaluated for neurotoxicity with the OECD test guideline 424. Rodents are most commonly used for these neurotoxicity studies that can be performed after acute, subacute, subchronic or chronic exposure. To evaluate the potential neurotoxicity, several parameters are studied, e.g. measuring of body weight, food/water consumption, functional tests as well as analysis of blood samples and histopathology. At the end of the study, a minimum of five animals per gender should be used to look at neurohistopathology in great detail. After that, the incidence and severity of the results should be correlated with the neurobehavioral and neurohistopathological effects. The collected results are meant to deliver a primary (tier 1) screening for potential neurotoxicity. In the case of a chemical screening positive during tier 1, it can be further evaluated (tier 2). Tier 2 usually employs behavioral tests (e.g. learning and memory tests), measurements of electrophysiological/neurochemical parameters (e.g. cell integrity, nerve conduction velocity, neurotransmission) and more extensive morphological studies.

Developmental neurotoxicity – definition and OECD test guidelines
On a regulatory level, systematic testing for DNT is not a standard requirement within the European union or the United States. DNT testing is only executed in higher tiered testing. Chemicals end up in tier 2 if they have triggered an alert based on chemical structure activity relationships or if there is evidence of neurotoxicity in systemic acute or repeated dose toxicity studies. As mentioned above, REACH states that the *in vitro* methods available today are not sufficient to determine DNT on their own. In 1991, EPA released the first DNT guideline (US EPA OPPTS Developmental Neurotoxicity Testing Guideline 870.6300 § 83-6), and began to assess some toxic compounds and pesticides. In 1998, EPA’s DNT study guideline was updated and revised. In 1995 the OECD started developing the OECD test guideline 426, using the EPA guideline as an outline. The OECD DNT test guideline (TG 426) was later enforced in 2007. According to the existing toxicity test guidelines, DNT is often examined *in vivo* using a large number of experimental animals, most commonly rodents. At least three different doses of the compound should be evaluated according to the test guidelines. The highest dose should result in maternal toxicity but the pregnant female cannot lose more than 10% of her body weight, and the lowest dose should not result in any noticeable toxic effects in the mother. The animals should be exposed from day 6 of gestation until postnatal day 10 according to EPA or postnatal day 21 according to the OECD guidelines. The pups are assessed from postnatal day 4 until postnatal day 75, a battery of tests are performed, i.e. clinical observations, bodyweight, brain weight, neuropathology, sexual maturation, behavioral ontogeny, motor activity, sensory function as well as learning and memory. Approximately 720 animals are needed to conduct a DNT study on a single substance. DNT testing has also been added to cohort 2 of the extended one-generation reproduction toxicity study (EOGRTS) (OECD Test No. 443). The EOGRTS was originally suggested as part of a tiered testing method for agricultural chemicals by the ILSI Health and Environmental Sciences Institute’s Agricultural Chemical Safety Assessment (ACSA) Technical Committee. They decided to add DNT and developmental immunotoxicity in the preliminary assessment of chemicals and removed the previously mandatory requisite for a second generation breeding studies. According to the EOGRTS, one male or one female from each litter should be selected for neurobehavioral testing and subsequent neurohistopathology assessment during weaning and one from each litter should be evaluated as adults. By adding DNT and developmental
immunotoxicity to the testing, the EOGRTS test better matched the increasing demands on studying these endpoints for agricultural chemicals. Although this battery of tests has been useful to identify DNT generating chemicals, it has major room for improvement. The current DNT test guidelines have been criticized for having relatively low sensitivity and results that can be difficult to interpret or in contrast, being too sensitive resulting in identification of many false positives. For example, a guideline study on a known DNT inducing chemical, methylmercury (MeHg), did not generate any adverse effects when looking at the classical endpoints in rats. MeHg only displayed adverse effects in this system when studying transcriptomic endpoints or when performing imaging analyses. Using the current guidelines, there are only 12 (13 counting valproic acid (VPA)) chemicals that are currently listed as DNT generating chemicals. Lead, mercury, arsenic, polychlorinated biphenyls, toluene, ethanol were all identified through epidemiological evidence. Since 2006, fluoride, manganese, tetrachloroethylene, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT) and polybrominated diphenyl ethers have been added to the list. It has been suggested that VPA should be added to this list based on clinical evidence. Taking all of this into consideration, in vivo DNT testing according to the current test guidelines is both time consuming, costly, requires a substantial amount of animals and is sometimes inaccurate in picking up DNT producing chemicals.

**OECD and adverse outcome pathways (AOPs)**

The concept of Adverse Outcome Pathways (AOPs) was first published in 2010 as a conceptual framework to support ecotoxicology research and risk assessment. In 2012, The OECD launched a new program that was focused on developing AOPs. This knowledge-based framework was built to help and support the toxicological risk assessment of chemicals. The incentive with this program was to standardize how an AOP was developed, reviewed, agreed upon and published according to OECD guidelines. The AOP development program is monitored by the Extended Advisory Group on Molecular Screening and Toxigenomics (EAGMST) and the members are nominated by National Coordinators. These members help with the internal review process as well as contributing actively in the development of AOPs. The online platform AOPwiki.org can be used to create an AOP of interest. This platform is constantly updated and refined as well as open access and aims to gather existing knowledge on how chemicals can induce adverse effects.

**Figure 2.** General structure of an Adverse Outcome Pathway (AOP). The AOP begins with a Molecular Initiating Event (MIE) followed by one or several Key Events (KEs) connected with Key Event Relationships (KERs) to yield in an Adverse Outcome (AO).
An AOP (Figure 2) displays existing evidence from literature regarding a chemical at different levels of biological organization that give rise to adverse health or ecotoxicological effects on a molecular, cellular, organ, organism and population level. The structure of an AOP is linear and starts with a molecular initiating event (MIE), by which the chemical first interacts with a biological target (e.g. by binding to a receptor). The MIE is followed by one or several key events (KEs) that lead up to the adverse outcome (AO) for that particular AOP. The AO should be at a level of biological organization that is relevant to toxicological risk assessment. The different KEs are connected by what is described as key event relationships (KERs) that describe the underlying correlation between the KEs. Table 1 lists examples of different MIEs, KEs (on both cellular and organ level) and AOs in relation to identifying chemicals that induce DNT in humans. By utilizing these platforms different research groups can easily share information and help speed up the process of toxicological evaluation of chemicals. There are currently several AOPs in the AOPwiki that have OECD status.

Table 1. Examples of events relevant for adverse outcome pathways (AOP) in the case of linking chemical exposure to DNT in humans. No relationship in the horizontal events. (Adapted with permission from Aschner et al., 2017)
Many AOPs are deficient in their quantitative features when it comes to chemicals, including chemicals that result in DNT. It is possible to link the events but it is unclear at which exposure doses this happens, or how much alteration at a MIE and a KE is needed for the AO that is displayed at the higher levels of biological organization. Because of this, more basic research is needed on the MIEs and the KEs induced by a substance. It has therefore been suggested that most of the newly developed DNT methods and models should aim at assessing the ability of a chemical to interfere with basic neurodevelopmental processes such as cell proliferation, differentiation, neuronal and glial cell migration, axonal and dendritic outgrowth, synapse formation and stabilization, apoptosis and myelination.13,71,72 There are currently open AOPs on the AOPwiki website for DNT such as the AOP 13 that describes that chronic binding of antagonists to N-methyl-D-aspartate receptors during brain development leads to impairment of learning and memory.73 There are also several others that are currently under development.

Evaluation of adult and developmental neurotoxicity in vitro

Neural proliferation, migration, differentiation, synaptogenesis, apoptosis and myelination are all important for neurodevelopment11 and more focus should be placed on these fundamental processes when establishing new model systems for DNT screening. There are multiple approaches that have been used in the past or that are currently being used to study these processes in vitro. The following section will give a brief summary of a few of these processes (endpoints) and give examples of assays that can be used to study them. Emphasis will be placed on the assays used in this thesis, however, more detailed descriptions will be given under the section “methodological considerations”. Cell lines, primary cultures or neural stem cells (NSCs) can be used to study the endpoints.

Proliferation

Proliferation is a crucial event in the brain and especially in the developing brain. Proliferation is important for the NSCs to be able to expand and give rise to the different structures of the brain.74 Disturbances to proliferation of NSCs is considered as key events for several neurodevelopmental diseases such as autism where one of the KEs on the organ level is an imbalance in the size and number of neurons in specific areas of the brain.75 There can be several MIEs that influence proliferation such as mutations or DNA histone modifications of genes involved in proliferation, alterations to contact inhibition pathways or energy restrictions.76 Example of DNT inducing chemicals that affects proliferation is methylazoxymethanol (MAM) that affects precursor cell proliferation77 but also chemicals like ethanol and toluene that have been shown to affect glial cell proliferation and maturation.78 Proliferation can be measured in different ways. There are metabolic cell proliferation assays such as tetrizolium based assays, e.g. 3-(4,5-Dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide (MTT),79 resazurin based assays, e.g. AlamarBlue80 or ATP detection through luciferase-detection.81 The metabolic assays are based on the same principle, i.e. the measurement of some aspect of general metabolism or an enzymatic activity. However, the metabolic activity does not necessarily correlate to proliferation in the assays mentioned above. Chemicals or events that induce oxidative stress, e.g. radiation, might give an increase in metabolic rate even though the cell number has stayed the same compared to control.82 When it comes to the tetrizolium based assay, the crystals that form need to be dissolved before readout and this makes this assay into a final endpoint assay. Resazurin based assays however can be used as a continuous endpoint throughout an experiment and is generally less expensive than tetrizolium assays. Another advantage of the resazurin based assays is that they can be
multiplexed with other assays, e.g. chemiluminescent assays to measure cytokines, apoptosis or gene/protein expression. It is therefore important to think carefully about what type of assay to choose for your experiments depending on other parameters you might want to study simultaneously. One should always be aware that the assays mentioned above do not distinguish between cell death and inhibited cell proliferation. To clarify the concept, if a chemical induces a reduction in the parameters mentioned above compared to control cells, it is not possible to determine if this reduction is due to cell death or if the number of existing cells are the same but that they simply proliferate at a slower pace or shift from mitochondrial to anaerobic respiration. However, you can combine these assays with a protein content assay and express results as metabolic activity per protein (which is proportional with the number of cells).

There are proliferation assays based on the incorporation of nucleoside analogs or radioactive nucleosides in to newly replicated DNA. These assays are generally considered very reliable and accurate. These assays include e.g. 5-bromo-2-deoxyuridine (BrdUrd) incorporation and $^3$H-thymidine incorporation. These assays measure the synthesis of newly formed deoxyribonucleic acid (DNA) by incorporation of detectable nucleosides or nucleoside analogs. $^3$H-thymidine is radioactively labeled thymidine where as BrdUrd will need detection with the help of labeled antibodies towards BrdUrd. Even though the $^3$H-thymidine incorporation assay has one less procedural step, one major disadvantage with this assay is of course the hazard of handling radioactive material. Proliferation can also be measured by antibody-detection of an antigen that is present during proliferation but not in non-proliferating cells, e.g. ki-67.

**Migration**

Migration of neurons is crucial when it comes to the developing brain. The developing neurons migrate from their sites of origin in the ventricular and subventricular zones to different areas of the developing CNS where they will differentiate and populate a particular structure. Regulation of the timing and direction of migration is highly ordered and disruptions to these patterns can have detrimental consequences for the developing brain. One example of a DNT inducing chemical that affects neural migration in children exposed *in utero* is MeHgCl, which causes intellectual disability. Both radial and non-radial migration of neurons has also been found to take place in the adult mammalian brain. There are many approaches to study cell migration. Effects of chemokines on migration can be investigated by using the trans-well migration assay, also called the Boyden chamber assay. In this method, cells are seeded in one of two compartments and chemokines are added to the other compartment. After fixation, it is possible to count the number of cells that have migrated over to the second compartment of the chamber. Another example is to use a cell-exclusion zone/migration gap closure assay. In this assay the cells are grown in one area but are restricted from entering another area by a physical barrier. The experiment starts once the barrier is removed and the migration of the cells can be monitored with real time microscopy. No chemoattractants are used in this assay. It is also possible to measure radial migration from adherent neurospheres. The results are presented as the distance that the precursor has migrated away from the attached neurosphere at a specific time point. It is also possible to perform single cell analyzes of cell migration through time-lapse microscopy.

**Synapse formation and function**

Synapse formation is a crucial event in the assembly of normal neuronal circuits in the brain. The formation and maturation process is dependent on proteins that are associated with the membrane but also secreted proteins. The process of synapse formation and strength is a dynamic process dependent on neuronal activity through stimulation of growth factor secretion.
and the localization of receptors both pre- and postsynaptically. Alterations to normal synaptic function are associated with both psychiatric disorders and neurologic disorders. It has also been shown to induce subtle AOs such as cognitive, psychomotor and sensory defects. Chemicals that interfere with KEs such as axonal transport to the nerve endings, the transcription or release of growth factors or basic neuronal activity can influence synapse formation/strength and can give rise to adult or developmental neurotoxicity. Astrocytes have also been found to influence synapse formation, function, and elimination, indicating that adverse effects in astrocytes might also have an impact on synaptogenesis. Examples of chemicals that have been shown to interfere with synaptic function, generating the AOs mentioned above, are organochlorine pesticides. Synapse formation can be studied by immunostaining or other protein detection assays of presynaptic proteins, e.g. synaptosomal-associated protein of 25 kDa (SNAP-25) or synapsin 1, or postsynaptic proteins, e.g. postsynaptic density protein 95 (PSD-95) or microtubule associated protein 2 (MAP2). It is also possible to measure neurotransmitter release by the use of fluorescent probes or fluorescent dyes like FM1-43.

**Apoptosis**

Ever since it was discovered that apoptosis is essential for normal brain development, the phenomenon has been extensively studied. Programmed cell death is detectable already during neural tube formation and continues throughout the development of the neural network. As mentioned above, the process of proliferation and migration are crucial processes for brain development, however, the process of apoptosis seems to be equally as important. Studies performed in mice showed that inhibition of apoptosis during brain development resulted in an abnormal increase in the number of neurons in several different neuronal subpopulations. Chemicals that interfere with neurotrophic growth factor levels, either at a transcription, translation, synaptic or receptor level can influence apoptosis. Several mutations in genes involved in apoptosis can also be important. The consequences of abnormal apoptosis can result in neuroanatomic abnormalities and developmental abnormalities resulting in cognitive disabilities. One example of a DNT inducing chemical that increases apoptosis in the developing rat brain is polychlorinated biphenyls. There are many ways to study apoptosis. DNA fragmentation assays and terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) are methods that monitor DNA fragmentation by incorporation of nucleotide analogues that can be labeled by antibodies. It is also possible to measure the activity of caspases by lysing the cells and adding a caspase substrate that generates a fluorescent, luminescent or colorimetric signal when it is cleaved. Annexin V has affinity for phospholipids that flip to the outer part of the cell membrane during apoptosis. Hence, fluorescently labeled annexin V can be used as a measurement of apoptosis. The fluorescent labeling makes annexin V visible under a fluorescence microscope or enables quantification by flow cytometry. The general process of cell death can be assessed using dyes that enter the permeabilized membranes of dead/damaged cells but are unable to pass through the membranes of viable cells. Propidium iodide (PI) is one example of such a dye that has been used in this thesis. PI enters late apoptotic and necrotic cells and bind to DNA with a stoichiometry of one dye per 4-5 base pairs of DNA and can be measured by fluorescence. The combination of annexin V and PI can be used to measure both apoptotic and necrotic cells. Live and healthy cells will be negative for both markers, whilst early apoptotic cells will be positive for annexin V and necrotic cells will be positive for both markers.
Differentiation

Neural differentiation plays an extremely important role in the developing brain. Several DNT inducing chemicals, e.g. organophosphate pesticides, have been shown to affect differentiation leading to AOs such as autism and cognitive impairments in population studies in humans. Transcriptional regulation is involved in the majority of the different processes of differentiation when it comes to exiting the cell cycle, fate determination, migration and expression of transcription factors or receptors. There are many examples of recommended markers for neuronal differentiation, e.g. neuron-specific enolases, neuronal nuclei, βIII-tubulin and doublecortin as well as markers specific for astrocytes such as glial fibrillary acidic protein (GFAP) and oligodendrocytes such as oligodendrocyte specific protein. It should be mentioned that mRNA evaluation does not necessarily reflect expression of functional gene products. Therefore, it is always good to perform both mRNA and protein assays if possible. mRNA can be analyzed by methods like polymerase chain reaction (PCR), RNA-sequencing and microarrays. Protein levels can be analyzed by methods like western blot, enzyme-linked immunosorbent assay (ELISA), flow cytometry or immunocytochemistry. It is also possible to study functional and structural parameters. Neurite outgrowth can be quantified by counting the number of cells with visual neurites, the amount of neurites per cell or by measuring the length or branching of the neurites. The quantification of neurites can be facilitated by staining the neurons with cell permeable dyes, e.g. calcein-AM. Calcein-AM is hydrolyzed to calcein after entering the cell, producing a fluorescent signal that allows the visualization of the cellular cytoplasm of live cells. The correlation between cell bodies and nuclei can be determined by using a nuclear staining, e.g. Hoechst. The use of staining procedures makes it possible to use different type of imaging programs to standardize and analyze results, which helps to speed up the process compared to manual counting. Methods like these are more suitable for high-throughput analyses. Functional endpoints can be studied by measuring the electrical activity of single neurons or in the entire culture/tissue as a unit. The cell membrane potential (CMP) can be measured by using charged probes that only enters the cells during depolarization. Inside the cells, the dye binds to intracellular proteins and emits greater fluorescence with a shift towards another spectrum. In this thesis, the FLIPR membrane potential (FMP) probe was used. The FMP probe is a slow-response probe but has been shown to be 14-fold faster than traditional slow-response probes.

Developmental neurotoxicity

The nervous system is particularly sensitive to toxic insult during development due to several reasons. As mentioned above, all different processes during development are important to generate the various brain structures. It is crucial that there is the right amount of cells with a specific function at the correct location for normal brain development. Synaptogenesis is a dynamic process, still active in the adult nervous system and the synaptogenic process that takes place during development is crucial to build the basic foundations and circuits. There might also be processes or proteins that have a slightly different or additional function throughout development compared to the adult nervous system. One such example is neurotransmitters that during development can modulate cell proliferation, survival and differentiation apart from their neurotransmission capabilities. This means that any chemical that disturbs neurotransmission during development may actually cause permanent damages to the CNS. It is clear from literature that it is not only the neurons that are sensitive during development, but also the astrocytes, oligodendrocytes and microglia. For example, it has been shown that several DNT
substances like ethanol and nicotine can cause extensive proliferation and increased maturation of glial cells if exposure occurs during the growth spurt of the brain.\textsuperscript{27,131} With all this in mind it is also important to remember that the brain of the fetus is not protected by a fully functional BBB. The development of the BBB is a gradual process that starts \textit{in utero} in the beginning of the second trimester in humans and continues until postnatal month 6.\textsuperscript{20}

Apart from what have been discussed above, there is also an emerging need for evaluating DNT since there is an increase in children being diagnosed with several learning and neurodevelopmental disorders over the past three decades.\textsuperscript{132–134} Neurodevelopmental disorders include attention deficit hyperactivity disorder (ADHD), autism, behavioral problems, learning disabilities, dyslexia, intellectual disability, fetal alcohol syndrome and schizophrenia, to mention the most significant. In the United States it has been showed that 17\% of children under the age 18 suffer from some kind of developmental disability.\textsuperscript{132,134,135} Apart from this, many neurodegenerative disorders affecting elderly persons are in fact believed to be neurodevelopmental diseases because the conditions share a common pathophysiological cascade involving oxidative stress and impaired hippocampal neurogenesis in mice.\textsuperscript{136,137} This further strengthens the need for good model systems to screen for DNT, since it is estimated that 35\% of all disease burden in Europe is related to brain disorders.\textsuperscript{138}

So what are the risk factors for neurodevelopmental disorders? It has been shown that direct and epigenetic alterations due to exposure to environmental insults, along with genetic and social factors, are risk factors (Figure 3).\textsuperscript{139,140} It is believed that it is a combination of these factors that contribute to the worldwide increase seen in developmental disorders.\textsuperscript{140} One could of course also argue that we have an increased awareness and understanding for these disorders, which have driven us to categorize and label the different conditions. The labeling of the disorders is a helpful tool when it comes to understanding and prescribing the correct treatment if such is necessary. A report from the NRC states that 28\% of all major developmental disorders in children are linked entirely or partly to environmental exposures.\textsuperscript{141} Three percent are a direct result of environmental exposure to chemicals and the other 25\% arise from interactions between environmental factors and specific genetic factors, making that particular individual susceptible.\textsuperscript{19}

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{figure3.png}
\caption{Risk factors associated with developmental neurotoxicity. The brain, the genes and the environment are mutually interactive domains. Adapted with permission from (Boivin et al., 2015).\textsuperscript{142}}
\end{figure}
As mentioned above, there are only 12 chemicals (13 counting VPA) that are currently listed as DNT generating chemicals: lead, mercury, arsenic, polychlorinated biphenyls, toluene, ethanol, fluoride, manganese, tetrachloroethylene, chlorpyrifos, DDT and polybrominated diphenyl ethers.\textsuperscript{19,65} Out of the ~80,000 chemicals registered at EPA, only ~200 chemicals have so far been tested for DNT.\textsuperscript{11}

**Neurotoxicity**

Neurotoxicity can be described as the adverse effects caused by a substance on a neural function or structure of the CNS or PNS. The toxicity occurs when there is an alteration in the baseline activity of the nervous system, resulting in permanent or reversible damages. Examples of such functions and complexes can be neurotransmission, neurotransmitter synthesis/degradation, neurotransmitter receptors or neurotransmitter reuptake. Substances that exert these adverse effects can be heavy metals, pesticides, drugs or naturally occurring substances (neurotoxins). The three neurotoxic compounds used in paper IV, i.e. atropine, nicotine and strychnine, are known neurotoxicants. The exert their toxicity through modulating the function of different neurotransmitter receptors, i.e. the muscarinic acetylcholine receptors (mACHR), the nicotinic acetylcholine receptors (nAChR) and the glycine receptors. Both nicotine and atropine are substances that interfere with acetylcholine receptors. Alterations in acetylcholine signaling can be lethal since it is the major neurotransmitter involved in parasympathetic signaling and in neuro-muscular transmission.\textsuperscript{143} Nicotine, being an nAChR agonist, activates the five subunit ion channel by binding at the two binding sites that are located in the interface between two α subunits or between an α subunit and a non-α subunit on the receptor, leading to stabilization of the open form of the receptor.\textsuperscript{144} The subunit composition of the receptor changes the affinity of different ligands, such as nicotine and acetylcholine, to the receptor.\textsuperscript{145} The ion channel is permeable to Na\textsuperscript{+}, K\textsuperscript{+} and Ca\textsuperscript{2+}.\textsuperscript{146} Atropine is an antagonist for the mACHRs, which are G-protein coupled receptors.\textsuperscript{147} Strychnine is a competitive antagonist for the glycine receptor, which is an ion channel permeable for Cl\textsuperscript{−} ions that is normally activated by glycine.\textsuperscript{148} The glycine receptor is one of the most abundant inhibitory receptors in the CNS and it is involved in the inhibitory neurotransmission in the spinal cord and brainstem. The glycine receptor also consists of five subunits and has four glycine ligand binding sites.\textsuperscript{148} Acute neurotoxicity caused by these classical neurotoxic compounds usually results in death by over- or under stimulation of the parasympathetic nervous system affecting the heart or breathing.

**In vitro models for DNT testing**

The idea of using *in vitro* tests to study toxicity, including DNT, has been increasingly recognized as the number of chemicals in need of evaluation increases. This was further emphasized with changes in legislations (e.g. REACH and the Cosmetics Directive (76/768/EEC) in the EU) that also press on the importance of the 3Rs regarding the use of animals in research and toxicity testing. The focus has slowly been shifted towards the development of alternative models, e.g. mammalian cell cultures or non-mammalian whole organism model systems like zebrafish (*Danio rerio*) or the nematode *Caenorhabditis elegans* (*C. elegans*). With the rapid development of *in vitro* methodology and the use of quantitative structure-activity relationship- (QSAR) based computational approaches, alternative methods could definitely be used in tier 1 to initially screen for potential neurotoxic/DNT inducing chemicals. This could help to narrow down the substances in need of further evaluations, which would speed the process along as well as spare animals and save money.
As mentioned, several of the processes involved in the development of the CNS can be studied using *in vitro* models. One reason why *in vitro* models are not yet sufficient for tier 1, is that there are some limitations related to toxicokinetic features such as metabolic capability, absorption routes, distribution and elimination (ADME). When using cell models there might also be intracellular and extracellular architectural changes combined with the loss of time and region dependent organization of cellular events. The following sections will cover some of the *in vitro* and non-mammalian methods that may be used to screen for DNT.

**Cell lines**

Cell lines have been used for a long time and they may serve as simple alternative models. The first established cell line dates back to 1943 when Wilton R. Earle created a cell line called L929 from the connective tissue of a mouse. The first human cell line was established in 1951 from a woman named Henrietta Lacks, who suffered from cervical cancer, hence the name HeLa cells. It is common that cell lines originate from tumors, since the cells are transformed and display specific characteristics. Tumor cells proliferate independent of growth factors, show no response to growth inhibitors, evade apoptosis, promote angiogenesis, display unlimited proliferation and are invasive.

The cell lines originating from a tumor all derive from one common ancestor cell that has expanded clonally. It is also possible to immortalize primary cells by using retroviruses or by the expression of telomerase reverse transcriptase protein (TERT). TERT expression is particularly useful in human cells since they are more affected by telomere length. Immortalized cells are not the same as transformed cells, since immortalized cells are dependent on growth factors and show sensitivity to growth inhibitors. The use of immortalization of primary cells broadens the possibilities in the field of cell line development since it is no longer limited to tumor cells. Hence, it is possible to take a biopsy from a tissue, immortalize the cells *in vitro* and continue to culture these cells as an immortalized cell line without being limited to the short lifespan of primary cells. More information about multipotent and pluripotent cell lines will follow in the section about stem cells.

The choice of cell line depends on the set up of the experiment and the endpoint of interest. One should always carefully consider the species, origin and the phenotypes of the cell line before making a decision. There are also some drawbacks to consider with using cell lines as a model. Cell lines are immortalized or originate from a tumor. Therefore, they do not necessarily behave as normal cells do in regards to phenotype and genotype. Transformed or immortalized cell lines express a high number of tumor growth-related genes that may affect the cellular response upon chemical exposure. Studies have also shown that cells with different passage numbers vary in their abilities to differentiate and can therefore react differently to the same chemical. Nevertheless, cell lines may be a fast, easy and simple way to study key mechanistic processes in neurodevelopment such as migration, differentiation, proliferation and synaptogenesis, providing that one is aware of their abnormalities and limitations.

The fact that the neural cell lines can differentiate into various phenotypes of neurons and glial cells further broadens the usage in toxicity testing. This enables toxicity testing of chemicals with diverse mode of actions (MOA). It is therefore important to be informed about what implications the particular differentiation protocol has and how that can influence the results. For instance, a chemical that inflicts its main toxicity through binding to the nAChR may be non-toxic to cells that do not express these specific receptors.
**Primary cells**

Different developmental processes, e.g. proliferation, migration, neurotransmission, receptor expression, apoptosis and differentiation can also be studied using primary cell cultures. Unlike most cell lines, primary cultures give rise to both axons and dendrites with functioning synapses. The functionality of both neuronal and glial cells can be maintained in primary cultures, making it possible to study neural and glial interactions. Primary cells are isolated from animal or human tissues and put into cultures for a limited period of time, usually for one experiment. Neuronal primary cells can be harvested from different regions of the nervous system but the two most common ones are cortex and cerebellum. Primary cultures usually generate a mixture of both neuronal and glial cells but neuronal cell cultures can be enriched by agents that block glia cell proliferation. Astrocytes have been shown to contribute to neurotoxicity and might influence the neuronal response to different toxic substances. Because of this reason, a mixed culture of both neurons and astrocytes might give a more complete model of the actual *in vivo* situation and be a more accurate indication of *in vivo* DNT.

The life span of primary cultures is shorter than for established cell lines since most primary cells do not possess the ability of self-renewal. Hence, new cultures need to be prepared for every experiment and will therefore lead to a lot of animal sacrifice. Historically, primary cultures have been the main model to further investigate mechanisms of toxicity observed in *in vivo* studies. However, there can still be pronounced differences between the results obtained from *in vivo* animal studies compared to *in vitro* primary cell cultures. The differences in outcome often originate from the fact that the cells may change function, appearance, cell-cell interactions and decrease their metabolism when removed from the host animal. Also, primary cultures consist of populations of post mitotic neurons that are not optimal for studying endpoints such as progenitor proliferation or fate determination. Cultured primary neurons from a specific region are committed to a lineage and often retain the properties of the region of origin when cultured *in vitro*. Lineage commitment can be useful when studying the effect of chemicals on different types of neurons or on a specific region of the brain. However, using primary neurons as a model for DNT screening of chemicals might be tricky since primary neurons derived from different regions of the brain have displayed conflicting results to the same chemical, e.g. during prenatal cocaine exposure in rats.

As mentioned earlier, the diverse functions and regions of the brain develop at different time points, which make certain chemicals toxic during different windows throughout development. For example, the DNT inducing compound MAM has different effects on the brain during different days of embryonic development. So in the terms of studying DNT, it is therefore important to consider when to isolate the primary cultures depending on what function you want to study and what time point during development you want to mimic *in vivo*. It is possible to isolate primary neurons at different stages of development, both from embryonic, fetal and postnatal tissues.

Over the past decade, the field of DNT research has shifted over to mostly using neuronal cultures derived from NSCs or neural progenitor cells (NPCs). These cells can be from human or animal origin, they are self-renewable and can be differentiated into several neuronal and glial cell types. The following section will further elaborate on this topic.

**Stem cells**

Stem cells are generally described as pluripotent cells that upon differentiation can give rise to any cell type in the body. Stem cells have the ability to self-renew and are commonly divided into two types: embryonic and adult stem cells. Embryonic stem cells originate from the inner
mass of the blastocyst and adult stem cells can be found in bone marrow, adipose tissue, and blood of the adult animal. Stem cells can also be harvested from the blood of the umbilical cord directly after birth. NSCs are somewhat further along the path of cell type commitment. They are no longer pluripotent but still differ from differentiated cells in three main ways: (i) NSCs have the ability to self-renew.\textsuperscript{171} (ii) NSCs are multipotent and can differentiate into all three neural lineages (neurons, oligodendrocytes and astrocytes).\textsuperscript{172,173} (iii) NSCs have the ability to regenerate neural tissue.\textsuperscript{174} NSCs can be isolated from different biological materials. They can also be generated through further differentiation of pluripotent ESCs derived from the inner mass of the blastocyst or be directly isolated from the blood of the umbilical cord or from the bone marrow as somatic/adult stem cells (SSCs). They can also be isolated from the subventricular zone and hippocampus in rodent brain as somatic/adult NPCs.\textsuperscript{176} Adult NPCs have not yet been proven to exist in vivo in humans.\textsuperscript{180} NPCs are similar to NSC but differ in the way that they cannot proliferate indefinitely, hence they will proliferate a limited number of times before becoming postmitotic.\textsuperscript{181} NPCs isolated as neurospheres from the brain cortex from a human male at gestational week 16 have also been used for DNT testing.\textsuperscript{163} Just as with different cell lines, ECSs, NSCs and NPCs have different ability to differentiate depending on the passage number, which can result in low reproducibility and inconclusive results without standardized protocols.\textsuperscript{123,182} The following section will cover some of the different types of pluripotent or multipotent cells that can be used for DNT testing.

ESCs are pluripotent and can therefore generate all cells from the three germ layers including neuronal and glial cells.\textsuperscript{183} Today it is possible to purchase both human and rodent ESCs from multiple companies. Because of their pluripotency, they must first commit to the neural pathway before they can further differentiate into the different neuronal and glial cells types.\textsuperscript{183–185} There are multiple protocols for the differentiation of ESCs. Different types of neurons can be obtained depending on the differentiation protocol, e.g. glutamatergic, dopaminergic, serotoninergic and GABAergic neurons.\textsuperscript{186–188} It should be mentioned that most of the protocols for ESC differentiation need optimization, mainly because ESCs can be difficult to keep in culture and the yield of fully differentiated neurons might be low.\textsuperscript{183,189} Optimizations of standardized protocols that are robust, reliable and affordable are needed.\textsuperscript{189} Human ESCs are preferable over murine ESCs since there is no need for extrapolating data between different species. However, human ESCs are in general more difficult to handle than murine ESCs.\textsuperscript{123} There is also an overwhelming ethical concern in using human ESCs that should be considered and different countries worldwide have distinct rules and regulations for the use of human ESCs.\textsuperscript{190} On the contrary, a good thing with using a mouse ESC model is that most of the available in vivo DNT data are from animal models which make a murine ESC model easier to compare to existing data.\textsuperscript{123} There are murine ESC models that have been validated as alternative models for in vivo DNT testing.\textsuperscript{191,192} The assays usually display shortcomings in the sense that they cannot detect toxicity to late developmental stages since the cells might not reach full maturity, but there are exceptions. Human ESC have been successfully used to study how chemicals affect neurogenesis\textsuperscript{193–195} and they have also been used to study neuronal maturation in the form of looking at neurite morphology in the means of neurite number, length or branching.\textsuperscript{196} Lancaster and coworkers created the cerebral organoid system in 2013 that consists of a human pluripotent stem cell derived 3D organoid culture system generated from neuroectodermal tissue.\textsuperscript{197} These organoids have been used to study the Zika virus,\textsuperscript{198} autism\textsuperscript{199} and brain development.\textsuperscript{200} Morphological attributes such as neurite length are generally considered as robust parameters to evaluate neuronal maturation and are applicable to high-throughput studies.\textsuperscript{201} There are no published studies looking at the DNT of neuronal network activity using human ESC. However, there are several systems under development that seem
promising. These systems have been used for acute neurotoxicity evaluations studying either synaptogenesis and/or neuronal electrical activity.\textsuperscript{202–204}

Pluripotent stem cells can be generated from all somatic cells by induction of four stem cell factors, i.e. Oct3/4, Sox2, c-Myc, and Klf4.\textsuperscript{205} Since this discovery about a decade ago, the research fields involving iPSCs have exploded. The use of iPSCs definitely opens up enormous possibilities that come with some great advantages. The donor cell is effortlessly and non-invasively attained without the need of embryonic tissue, which circumvents the ethical issue of using human ESCs. In the case of medical treatments of patients, there is early research on generating iPSCs from the patient’s own somatic cells.\textsuperscript{206} This can for example reduce the risk of rejection of a transplant. The personal iPSCs could also be used for individual disease modeling since they carry the same genetic disease-causing feature as the patient. The use of iPSCs could also open doors to personalized screening of patients for specific susceptibility to certain chemicals, such as pesticides.\textsuperscript{207} iPSCs are not recommended to study adult neurotoxicity since they do not reach a fully differentiated state that corresponds with adult brain physiology, even after three months of cultivation.\textsuperscript{208} However, during the last couple of years there has been an increased emphasis on using iPSCs for DNT screening. It has been suggested that currently available human neuronal and glial iPSC models could be used to gain mechanistic insight that could support different regulatory applications.\textsuperscript{209} This is needed due to the fact that the triggers that would qualify a chemical for tier 2 testing are based on observations made in adult rodents, and might actually not be good indicators for DNT. Hence, it is of great relevance to develop alternative models that could more accurately identify DNT triggers. Human iPSCs are suggested as the model of choice as they allow for the evaluation of many key developmental processes since they can mimic different windows of exposure.\textsuperscript{210} There are also limitations to using iPSCs. When comparing ESCs with iPSCs it has been shown that there are some changes in gene expression and DNA methylation patterns between the two cell types.\textsuperscript{211} It has also been shown that the iPSCs have similar DNA methylation pattern as the donor somatic cell, which would indicate that the iPSCs have a residual epigenetic memory that might influence the cellular response.\textsuperscript{212} Like with immortalization of any cell type, there is also a risk of insertional mutagenesis when using virus-based vectors for gene delivery.\textsuperscript{213} It has also been shown that human iPSC-derived models usually display a lower ratio of glial cells compared to the developing brain \textit{in vivo}, which might also affect the response to chemicals.\textsuperscript{214} Nowadays, there are iPSC cell lines that can be purchased, either in their naïve pluripotent stage or semi-differentiated. There are also standardized protocols for differentiation, making the use of these cell lines more robust and reproducible between different laboratories.\textsuperscript{215}

As mentioned briefly above, NSCs can derive from different origins. Human umbilical cord blood derived NSCs are SSCs that are derived from blood from the umbilical cord. Advantages with these stem cells are that they have similar characteristics as ESCs but there is far less ethical concern involved.\textsuperscript{216} Differentiation of human umbilical cord blood derived SSCs have showed generation of glutamatergic, dopaminergic and GABAergic neurons\textsuperscript{217–219} and have been used successfully for DNT testing.\textsuperscript{220,221} Bone marrow derived stem cells are SSCs that have the ability to self-renew and they can differentiate with the help of inducing factors into both neurons and glial cells.\textsuperscript{222} There is a lower frequency of SSCs in the adult bone marrow compared to human umbilical cord blood and the cells can be somewhat difficult to isolate and expand.\textsuperscript{223} Differentiation of bone marrow derived SSCs have showed GABAergic and serotonergic neurons.\textsuperscript{224,225} Differentiation has also yielded electrically active nerve cells with properly working ionic currents and calcium channels.\textsuperscript{222} Taking all this into consideration bone marrow derived stem cells could be suitable for DNT testing.
Non-mammalian alternative models

As mentioned before, the development of the nervous system is extremely complex and the limitations of simple *in vitro* models has raised the concern that they will not be able to predict DNT as well as whole animal *in vivo* models.\(^71\,149\) Due to the fact that a lot of the neurodevelopmental processes are conserved across different species, non-mammalian models such as the sea urchin, *C. elegans* or *Danio rerio* (zebrafish) have been successfully used to study the development of the nervous system. These models could be considered good candidates for alternative DNT testing.\(^71\,226\) However, there are limitations connected with non-mammalian models as well. Many of the simple organisms lack specific organs, e.g. kidneys, liver, lung, skin and a circulatory system. In some contexts, when studying complex conditions, the evolutionary distance from humans might be problematic. Also the fact that many of these species have the ability to self-fertilize, which results in fewer males in wild type population, can make it difficult to study sex-specific differences.\(^227\)

Sea urchin

The sea urchin is one of the most well studied non-mammalian species for DNT testing. Its developmental processes have been studied for a long time and are well known.\(^228\text{–}231\) Neurodevelopment of the sea urchin is dependent on many different neuronal phenotypes similar to humans, such as serotonergic, cholinergic, dopaminergic and monoaminergic neurons.\(^228\text{,}230\text{,}232\) The sea urchin is not only homologous to humans in many functions, but it is also suitable for high-throughput screening of toxic compounds due to its rapid development. The adult specimen can be kept in a tank from which gamets can be harvested and fertilized. The embryos and larvae can be cultured while the different parameters of DNT can be studied.\(^233\) Some of the advantages of the model is that the sea urchin spawn eggs that are transparent, which makes it possible to detect developmental malformations with a simple light microscope.\(^228\text{,}229\text{,}231\) The sea urchin has been used to study some developmental neurotoxicants such as chlorpyrifos, nicotine and organophosphate insecticides that targets the cholinergic system.\(^232\text{,}234\text{,}235\)

*Caenorhabditis elegans*

*C. elegans* is also highly (~80%) homologous to humans.\(^236\) The non-parasitic nematode only has 302 neurons (383 in males) and the complete map for its chemical and electrical connections are known; there are 118 distinct classes of neurons and 56 glia cells, which together form over 7600 synapses.\(^237\) The nematode has been an attractive experimental non-mammalian model due to many advantages: small size, short life cycle, ability to self-fertilize and high reproductive rate (>300 offspring per hermaphrodite). All of these attributes make its maintenance in the lab relatively easy, inexpensive and suitable for high-throughput testing of chemicals. The nematode is also transparent which makes microscope analyzes possible by using reporter gene fusions with for example fluorescent proteins that enables visualization of cell morphology and protein expression patterns *in vivo*.\(^238\text{–}240\) The nematode neurons signal by classical neurotransmitters such as glutamate, GABA, DA, 5-HT and ACh.\(^241\) The nematode has been explored as an alternative candidate for DNT testing and has been used to study DNT inducing chemicals such as manganese, MeHg and arsenic.\(^242\text{–}244\)

*Danio rerio* (Zebrafish)

The zebrafish originates from Southeast Asia and it is one of the best described and studied models for studying developmental biology.\(^245\) The zebrafish embryo is also transparent which comes with all of the benefits mentioned in previous sections and the *ex utero* fertilization also makes it easy to study different dose-dependent effects at different developmental stages. The
zebrafish also shares some homology (~70%) with humans. The development of the zebrafish is rapid (the basic CNS and patterning of sub-regions of the brain is completed three days after fertilization) which makes it suitable for high-throughput studies. There is also physiological similarity to humans and other vertebrates in which biological barrier systems are developed. The zebrafish has a BBB that is functional for large molecular weight molecules (10 kDa) already 72 hours post fertilization. However, smaller molecule tracers (<1 kDa) were not excluded until 5-10 days after fertilization. It is possible to study the development of specific neurons and axons live using a light microscope due to its very rapid embryonic development. Another advantage with the zebrafish model is that there is no ethical permit required for studies performed up until 5 days after fertilization, since it is regarded as independently feeding by its yolk sac up until that point. It is also possible to study behavioral endpoints by observing swimming patterns of the zebrafish. The zebrafish model has been used to study the DNT effects of many known DNT producing chemicals, including arsenic, MeHg and lead. The fact that it is possible to study neurons and axons in real time after addition of a toxic compound makes the model an interesting candidate for DNT testing.

In conclusion, these non-mammalian model organisms are small in size, have high availability, short generation times and low cost compared to e.g. rodents. These attributes make these models favorable for high-throughput DNT screening.

Pathways involved in neuronal differentiation

There are many different markers and signaling pathways important for neural differentiation. The following two sections will elaborate on the signaling pathways that are discussed in paper III, i.e. cAMP response element binding protein (CREB) activated gene transcription, brain-derived neurotrophic factor (BDNF) signaling and RA signaling.

CREB-dependent gene expression

CREB was first identified in 1987 as a nuclear protein that bound to the cAMP response element (CRE) of the somatostatin gene in PC12 cells. There are three isoforms of CREB (alpha, beta, delta) all transcribed from the CREB1 gene. The alpha isoform is the most common one, however, all isoforms are important for neuronal function in different parts of the brain or during different developmental stages. Due to its structure, CREB can form homo- or heterodimers after which it can interact with DNA, cofactors and the basal transcriptional complex. CREB consists of several regions that are responsible for the many different binding targets of CREB. The KID region of CREB connects intracellular signaling events to CREB activity since it includes a Serine 133 (Ser133) that is widely targeted by many activity-induced kinases such as Ca²⁺/CaM-dependent kinase II and IV, protein kinase A (PKA), protein kinase C (PKC), ribosomal S6 kinase (RSK) and protein kinase B (AKT). This means that different signaling pathways can phosphorylate Ser133, including increased intracellular Ca²⁺ and cAMP. Furthermore, CREB can be phosphorylated on several other serine locations, e.g. Ser142 and Ser129, by other kinases. Phosphorylation at these locations results in somewhat contradicting outcomes by both enhancing and suppressing CREB-regulated gene expression. CREB can also be dephosphorylated by Serine/Threonine-specific protein phosphatases type 1 and 2A, which in turn represses gene expression. The main mechanism in which CREB activation is regulated is via the phosphorylation of Ser133, but it has been reported that CREB can be regulated by other mechanisms such as acetylation, ubiquitination, sumoylation, and glycosylation. CREB-activated gene expression can
also be regulated by epigenetic regulation such as methylation of the CRE binding site that has been shown to repress CREB-dependent gene expression. Other chromatin remodelers, such as the histone acetyltransferases, CREB-binding protein and p300, have also been shown to influence gene expression. These processes have been shown to play a role in the inducible expression of BDNF in the CNS.

A lot of effort has been put in to determining which genes are regulated by CREB. With the help of modern techniques, it has been established that CREB is involved in the regulation of a wide array of both inducible and constitutively expressed genes. Among these are a great number of genes related to neuronal function, such as genes coding for neurotransmitter receptors, e.g. acetylcholine receptors Unc-38, Acr-7, Acr-8 and Lev-1, the glutamate receptor Glr-6 and the dopamine receptor Dop-2. From these studies, it is clear that CREB serves an important role for neuronal function, survival, proliferation and differentiation. Creb1 null mice died directly after birth and pathological evaluation showed severe defects in their CNS, including fewer neurites. Interestingly, contrary to what was seen in the PNS, there were no signs of increased apoptosis in the CNS. This was explained by an upregulation of cAMP response element modulator (CREM) in these regions that would partly compensate for the absence of CREB. Mantamadiotis and coworkers confirmed this theory in 2002 when they deleted both Creb1 and Crem which resulted in increased apoptosis together with a decrease in the number of neuronal and glial precursors in the CNS. The increase in apoptosis and reduced neuronal differentiation seems to be caused by the inability of neurotrophic factors like NGF and BDNF to stimulate the survival, for instance by expression of anti-apoptotic gene Bel-2, and axonal growth programs dependent on CREB activation. Hence, CREB is important both for survival of post-mitotic neurons but also for the neural differentiation process. Even though CREB has an important role for neuronal development, there is a crucial balance to CREB-mediated gene expression. Increased CREB-mediated gene expression in mice resulted in negative implications for the brain. It was shown in a transgenic mouse model that over-activation of CREB gene expression caused epileptic seizures and resulted in cell death via an excitotoxic mechanism rather than a pro-apoptotic mechanism as with CREB inactivation. Interestingly from a pathophysiological standpoint, it has also been shown that CREB contributes to neuronal survival by regulating expression of antioxidant genes such as heme oxygenase-1, peroxisome proliferator-activated receptor gamma coactivator-1α and manganese superoxide dismutase. Hence, CREB activated gene expression may play a role in ROS-mediated neurotoxicity. Many chemicals that induce DNT have been shown to influence the levels of phosphorylated CREB, e.g. organophosphates and lead.

**BDNF signaling in neurons**

As mentioned above, CREB can be activated by different signaling pathways. One of them is via BDNF binding to the tropomyosin receptor kinase B (TrkB) receptor. BDNF can also bind to the p75 neurotrophin receptor (p75NTR). This next section will focus on the BDNF signaling in neurons and its correlation with CREB.

The neurotrophic factor BDNF was shown to promote survival in dorsal root ganglion cells in 1982 and was purified shortly thereafter. BDNF has since then become one of the most well studied neurotrophins due to its many complex roles in neuronal survival, neurogenesis and neuronal differentiation. The structure and the regulation of the BDNF gene is complex. It consists of 9 different promoters that can produce 24 transcripts, all of which are translated
to the same dimeric protein in its mature form. The regulation of BDNF gene expression takes place on a multilevel scale where different BDNF promoters can be found at different locations and in different tissues, generating a tissue-specific expression of different BDNF transcripts. It has been suggested that different exons might influence translation efficiency or stability of the transcript and thereby affect the availability and intracellular targeting of BDNF. The existence of non-coding antisense transcripts has also been found which might contribute to an additional level of regulation of BDNF mRNA translation.\textsuperscript{286} Once transcribed, BDNF is translated into the precursor protein pre-pro-BDNF and further processed into pro-BDNF in the endoplasmic reticulum. Pro-BDNF is a 32-kDa protein that can be proteolytically cleaved by intracellular proteases into the 14 kDa mature form of BDNF (mBDNF) that is thereafter secreted from the cell by exocytosis. Pro-BDNF can also be released from the cell where it can act extracellularly in its pro-form or be cleaved into its mature form by extracellular proteases.\textsuperscript{287} Even though it is not exactly clear from the literature, it seems like extracellular pro-BDNF is more abundant than mBDNF.\textsuperscript{288} But nevertheless, both pro-BDNF and mBDNF are active signaling proteins and can bind to receptors extracellularly. BDNF mRNA can be located both pre- and postsynaptically and can be transported by retrograde and anterograde transport to the soma as well as to proximal and distal dendrites, where it is locally transcribed in a neuronal activity dependent manner.\textsuperscript{289,290} Once pro-BDNF or mBDNF is secreted, it can act both in an autocrine and paracrine way depending on the receptor expression of the site of its release.\textsuperscript{291} Hence, locally translated mBDNF and pro-BDNF can be secreted and influence synaptic transmission and synaptogenesis. Neuronal activity has been shown to influence the translocation of both the BDNF mRNA and the BDNF proteins to the dendrites.\textsuperscript{290,292} As mentioned in the beginning of the section, BDNF can bind and activate two different receptors both pre- and postsynaptically, i.e. the TrkB and p75NTR receptors. mBDNF has higher affinity for the TrkB receptor compared to pro-BDNF, whereas pro-BDNF preferably binds to the p75NTR receptor.\textsuperscript{293} The cellular response that follows activation of these receptors can be dependent on where the receptors are expressed. Furthermore, other factors such as the recycling via endocytosis of the BDNF protein can modify the BDNF signaling response. This process seems to be dependent on the synaptotagmin isoform 6, which is independent on the synaptotagmin isoforms involved in secretion of mature and pro-BDNF.\textsuperscript{294} Figure 4 shows a simplified schematic illustration of the different BDNF signaling pathways. It has also been shown that there are splice variants of the TrkB receptor that do not contain the tyrosine kinase domain.\textsuperscript{295} These TrkB receptors lack the ability to generate a downstream signaling after binding of mBDNF and dimerization. These receptors can act as an inhibitor of BDNF signaling as the splice variant can dimerize with a full-length TrkB, bind mBDNF and internalize the entire complex, hence acting as a clearance receptor.\textsuperscript{296}
In general, all major signaling cascades that follow mBDNF activation of the TrkB receptor stimulate Ser133 phosphorylation of CREB and activation of CREB-dependent gene expression. There are multiple feedback mechanisms involved in the control of BDNF signaling. Interestingly, it has been shown that CREB binds to CRE elements in the BDNF gene promoter and hence stimulates the transcription of BDNF itself. BDNF signaling has also been shown to be able to regulate its own release as well as increase the surface expression of TrkB receptors. These self-induced feedback mechanisms most likely strengthens and stabilizes the generation of synaptic connections, which may be an explanation to why BDNF has been found to promote long-term potentiation that is associated with improved long-term memory. There are also negative feedback mechanisms regulating BDNF signaling. Excessive BDNF release leads to a decrease in the number of TrkB receptors at the neuronal cell surface which generates a long-term desensitization to BDNF both pre- and postsynaptically. Apart from the activation of CREB-mediated gene expression, BDNF has an influence on membrane excitability and synaptic transmission. For example, BDNF has been shown to affect the release of glutamate and GABA presynaptically by its activation of the RAF-MEK-ERK pathway where ERK has been shown to phosphorylate synapsin. BDNF also generates an increase in the number of neurotransmitter vesicles that are docked at the active zones in the synapses. BDNF regulates features postsynaptically by altering the activation kinetics of NMDA and GABA receptors, thereby affecting both excitatory and inhibitory neurotransmission. It also seems like BDNF, through ERK signaling, increases
excitatory transmission by increasing the translocation of AMPA receptors to the presynaptic surface. Some DNT chemicals have been shown to affect BDNF/TrkB signaling, e.g. fluoride, perfluorooctane sulfonate and organophosphates.

**All-trans retinoic acid signaling**

All-trans-retinoic acid (RA) is an essential molecule that forms in the body as a metabolite of retinol (vitamin A) oxidation. The importance of vitamin A for adult life has been known for a very long time. Already 3,500 years ago, the ancient Egyptians treated night blindness by eating beef liver (which is rich in vitamin A). Since then, it has been established that RA is important for several other functions such as renewal of skin epithelium, spermatogenesis and for a functioning immune system. During embryogenesis, RA functions as a morphogen that regulates the formation of the anterior/posterior axis. It does so by activating the transcription of different genes important for the vertebrate body plan, e.g. the homeobox genes (HOX), which are transcription factors that are extremely important for embryonic development. The function of RA is highly concentration-dependent and both excess and insufficient levels of RA result in malformations. Several malfunctions have been seen in both rodent and zebrafish studies where they have used genetic loss-of-function of RA synthesis or the RA receptors. Furthermore, when it comes to neural cells, RA has been shown to be important for neural differentiation, motor axon outgrowth and neural patterning in many different model systems.

![Figure 5](image.png)

**Figure 5.** The pathway of all-trans retinoic acid (RA) biosynthesis and degradation. Retinol is reversibly oxidized to retinaldehyde by the retinol dehydrogenase RDH10, followed by an irreversible oxidation to RA by the retinaldehyde dehydrogenase (RALDH1, RALDH2 or RALDH3). Retinaldehyde can also be reduced back to retinol by the dehydrogenase reductase DHRS3. RA is catabolized to inactive metabolites by members of the cytochrome p450 family (e.g. CYP26A1, CYP26B1 and CYP26C1).
RA can be produced from retinol by two steps of oxidation (Figure 5). RA is the major metabolite while isomers like 13-cis-retinoic acid is produced in much lower quantities and has totally different functions since it acts as a chromophore in vision. Retinol is first reversibly oxidized by retinol dehydrogenases (RDH10 and possibly others as well like RDH16) to retinaldehyde. The second step is that retinaldehyde is irreversibly oxidized by three different types of aldehyde dehydrogenase/retinaldehyde dehydrogenase (ALDH1A1/RALDH1, ALDH1A2/RALDH2, and ALDH1A3/RALDH3) to RA. The reduction of retinaldehyde to retinol is performed primarily, but not exclusively, by a short chain dehydrogenase reductase called DHRS3. RA can be catabolized by several members of the cytochrome p450 family (e.g. CYP26A1, CYP26B1, CYP26C1), processes that are important to prevent toxicity from excess of retinol. Since RA is the active metabolite involved in transcriptional activation, it is dependent on the cells’ ability to metabolize retinol to RA. However, RA can be released and internalized by adjacent cells and RA signaling is therefore considered as paracrine signaling in all cases, except during spermatogenesis. In the cytoplasm, RA is associated with the cellular retinoic acid-binding protein (CRABP), which delivers RA to the nucleus. In the nucleus, RA can bind to nuclear RA receptors (RARs), which form heterodimers with the retinoid X receptor (RXR). The RAR-RXR heterodimer complexes can bind to specific RA response elements (RAREs) that are located in the enhancer regions of certain genes. There are three RARs (α, β and γ) and three RXRs (α, β and γ) identified so far in most vertebrates. All of the RARs and RXRs can have several isoforms, generating a wide array of possible combinations of the receptors. With a few exceptions during the absence of RA, the RAR-RXR heterodimers bind to the RAREs and recruit histone deacetylase (HDAC) protein complexes that lead to histone H3 lysine 27 trimethylation (H3K27me3) and subsequent gene silencing. On the contrary, presence of RA induces a conformational change in the RAR-RXR heterodimer complexes that leads to the recruitment of co-activators that recruit proteins that mediate histone H3 lysine 4 trimethylation (H3K4me3) that relaxes the chromatin, which leads to gene activation.

Due to its various functions throughout development and adult life, it is clear that internal levels of RA are extremely important. Adverse effects in the form of craniofacial, heart, and nervous system malformations can be seen if RA is present at either too high or too low concentrations. For this reason, the RA signaling pathway is of interest when it comes to evaluating the MOAs of DNT inducing chemicals such as phthalate esters.

**Neurotoxic and developmental neurotoxicity producing chemicals**

As mentioned previously, many substances including drugs, industrial chemicals and pesticides have been proven to be neurotoxic or give rise to DNT. The following section will give a brief summary of the chemicals that were used to study neurotoxicity or DNT in the studies included in this thesis as well as cover the basic fundamental aspects of choosing positive and negative control compounds. Acrylamide (ACR) will be the main focus since it covers a greater section of this thesis.

**Implications in choosing positive and negative control compounds**

The process of selecting positive and negative control compounds for DNT studies is not trivial. In regards to selecting a positive control compound, there is usually a delay in the manifestation of the AO of a chemical interfering with a neurodevelopmental process. Because of this, it is
difficult to select a positive control compound that will reflect all of the biological processes relevant for DNT. Hence, if a chemical is not picked up by the test system it does not necessarily mean that it is not a DNT producing chemical. For example, a test system that measure neurite outgrowth would not identify the established DNT inducing chemical MAM as a DNT inducing chemical. The reason for this is that MAM affects precursor cell proliferation and not neurite outgrowth. It is also problematic if it is the other way around, i.e. if a chemical that is not considered as a DNT in vivo is picked up as a DNT in the test system. One example of this would be if MAM, that affects proliferating cells, is picked up in a system that evaluates neurite outgrowth. MAM would then be considered a false positive. However, it could just as well be a true positive hit in that MAM could affect a different target that in vivo systems have failed to picked up due to their high level of noise or low sensitivity. Due to these difficulties it has been suggested that a battery of positive control compounds including the “golden standard of DNT chemicals” should be used to minimize these issues. It has also been suggested that endpoint-specific control chemicals should be used (also referred to as “endpoint-selective controls” or “mechanistic tool compounds”). These positive control compounds would consist of chemicals known to affect the specific endpoint that is studied in that particular test system (examples of such endpoints can be found in Table 1). The choice of negative controls is equally as important as a positive control when it comes to evaluating a test system. The negative control should not affect the test system and should hence have no effect on the neurodevelopmental processes involved or the pathways that are behind these processes. Ideally, the negative control compound should have an adverse effect or at least be active in another system, but have no effect on the particular endpoints being studied in the system in question. When it comes to studying DNT, it is difficult to find pharmacologically active compounds for other systems that do not induce any DNT effect. In these situations, it is common to use negative control compounds that do not cross the cell membrane (such as mannitol that is used in this thesis). Chemicals that target other organ systems are generally also thought of as good negative controls, e.g. if studying the nervous system, a liver toxicant such as paracetamol can be used. However, although paracetamol is negative in most systems up to mM levels, it has been debated to be developmentally neurotoxic in vivo. Negative controls can also be chemicals with a molecular target that is not expressed in the system, e.g. omeprazole if the system does not express proton pumps. When it comes to negative controls for DNT, drugs that are allowed during pregnancy are generally considered as good choices.

**Acrylamide (ACR)**

ACR is a Group 2A probable carcinogen and a well-known neurotoxic compound. It is a water-soluble, vinyl monomer that has various applications in the chemical industry. Polymerized ACR, which is considered as non-neurotoxic, is used for water management, dye synthesis, in textile additives and in paint softeners as well as in the cosmetic industry. ACR can also be generated from food components during heat treatment as a result of the Maillard reaction between amino acids (mainly asparagine) and reducing sugars or reactive carbonyls. In Sweden, ACR is probably mostly known for being the neurotoxic compound in the sealant called Rhoca-Gil that was used to plug leaks in a tunnel construction being drilled through the Hallandsås in 1997. The polymerization process of ACR failed and monomeric ACR contaminated wells with drinking water and creeks. As a result, farmers at the Hallandsås reported that their cows became paralyzed and died and dead fish were floating in the water of the creeks. Tunnel workers and people who lived nearby also reported that they suffered from numbness due to neurotoxicity.
Absorption and distribution
Most people are exposed to ACR through food consumption and environmental pollutions. Studies on exposed populations and laboratory animals have shown that ACR can be absorbed orally, dermally, and by inhalation. Regardless of the absorption route, ACR is distributed throughout the body. The tissues most susceptible to ACR include liver, kidney, smooth and skeletal muscle, and the nervous system.

Metabolism and excretion
There are different routes for ACR metabolism and excretion (Figure 6). ACR can either undergo phase II conjugation with glutathione, a reaction catalyzed by glutathione S-transferase. This is followed by degradation of the glutathione adduct leading to the formation of the metabolite N-acetyl-S-(2-carbamoyethyl) cysteine which can be easily excreted. This mercapturic acid metabolite has been found in the urine of exposed mice as well as humans and have successfully been used as a marker for ACR exposure. The conjugation to glutathione is the most common metabolic pathway for ACR in humans. There are some differences in terms of the metabolism of ACR, both in the terms of interspecies differences but also individual differences within a species. These differences seem to be related to the activity and/or genetic polymorphisms of different metabolic enzymes involved in the metabolism of ACR. All metabolic pathways are present in both rodents and humans but to different extents. In rats for example, the most common metabolic pathway is through a member of the cytochrome P450-family called CYP2E1. CYP2E1 is a phase I enzyme introducing reactive or polar groups to xenobiotics. The CYP2E1-mediated epoxidation of ACR generates a second metabolite, glycidamide. At low doses, 50% of ACR is metabolized to glycidamide. However, at high doses, the CYP2E1 pathway gets saturated leaving only 13% of ACR being metabolized to glycidamide. The extent of ACR epoxidation in different species is reported to be rat > mouse > human.

![Figure 6](image-url)

Figure 6. A proposed scheme of ACR metabolism showing the formation of glycidamide, glutathione conjugates and hemoglobin- and DNA adducts. Image adapted with permission from (Ghanayem et al., 2005).

Both ACR and its metabolite glycidamide have been found to induce toxicity both in vitro and in experimental animals. In comparison to ACR, glycidamide acts more like a hard electrophile,
making it DNA-reactive and thus, a possible carcinogen. Both ACR and glycidamide can bind to plasma proteins by primarily reacting with the thiol group of cysteine or the amino group of the N-terminal valine in hemoglobin. ACR-hemoglobin adducts have been measured in both humans and rodents that have been exposed to ACR. Hemoglobin adducts in the blood are often used as a measurement of ACR exposure.

ACR and its metabolites can be excreted rapidly from the body by biphasic elimination with a 1st component t\textsubscript{1/2} of <5 hours and a 2nd component t\textsubscript{1/2} of 6-8 days. Nevertheless, ACR and its metabolites have the ability to accumulate in the body by covalently binding to proteins. This accumulation might be one of the reasons for the neurotoxic effects produced by ACR. \(\alpha,\beta\)-unsaturated carbonyl compounds like ACR are considered to be soft electrophiles that will react most favorably with soft nucleophiles such as the sulfhydryl groups on cysteine residues in proteins. The electron deficient double-bond reacts spontaneously with nucleophiles (mostly hydroxyl-, amino-, or sulfhydryl- containing compounds) by Michael addition. Due to this, ACR will react rather unspecific with different proteins, making it more difficult to predict the MIE for the neurotoxicity. However, the Michael addition to the thiol group of glutathione appears to be the most efficient reaction.

**Neurotoxicity of ACR**

ACR has been a well-known neurotoxic substance since the 1950s when it was realized that occupational exposure could give rise to cumulative neurotoxicity characterized by ataxia, skeletal muscle weakness, cognitive impairment, and numbness of the extremities. Since the mid-1960s it has been shown in animals as well as in tissue- and cell cultures that the monomeric form of ACR gives rise to similar neurotoxic effects as seen in exposed human populations. The monomeric form has been found to not only induce neurotoxicity but also reproductive toxicity, genotoxicity and carcinogenicity. However, only ACR-induced neurotoxicity has been documented in occupationally exposed populations in humans, indicating implications in human health.

From experiments performed in animal models, it has been suggested that ACR-induced neurotoxicity is mediated through axonopathy in both the central and peripheral nervous system caused by initial nerve terminal damage and subsequent retrograde axon degeneration. It has been shown that the neurotoxic effects of ACR are dose-dependent and they accumulate over time. It seems like it is not the accumulation of ACR, but the accumulation of damages over prolonged exposure that leads to neurotoxicity. The mechanisms underlying the neurotoxic effects of ACR are not fully known and they have been debated for a long time. Historically, there are three major hypotheses for the mechanisms underlying ACR induced neurotoxicity that have been the focus of most papers published within the field (Figure 7). The first one is the inhibition of kinesin-based fast axonal transport by reducing the affinity between the microtubules and kinesin to each other through covalent modifications. If axonal transport is affected there will be attenuated neurotrophic factor support at the nerve terminals. Impaired anterograde axonal transport could also affect the number of mitochondria at the nerve terminal, giving rise to energy depletion. This could result in “dying back” of the nerve body, mediated through the distal axonal degeneration. During development, the transport of pivotal axonal and dendritic building blocks could be affected, resulting in impaired neurite outgrowth. The second hypothesis is the alteration of neurotransmitters, their metabolites, neurotransmitter receptor density and affinities in the CNS. ACR has been reported to change the number and sensitivity of dopamine and serotonin receptors as well as mAChRs in the striatum of orally exposed rats. The third hypothesis is that ACR directly inhibits neurotransmission through the interference with the membrane fusion process at the nerve terminal, leading to
failure of synaptic vesicles to fuse with their target membranes. ACR readily form adducts with the SNARE protein synaptosomal nerve-associated protein 25 (SNAP-25) and N-ethylmaleimide sensitive factor which results in inhibition of these proteins and hence, subsequent reduction in neurotransmitter release. ACR has been suggested to react with, and inhibit, several proteins in the nerve cell, e.g. vesicular ATPase and the dopamine transporter, which probably further contributes to axonal degeneration or dysfunction. ACR has also been shown to reduce the uptake of dopamine into striatal synaptic vesicles. ACR increases the intracellular free Ca\(^{2+}\) concentration, which activates the proteolytic enzymes calpains. Concomitant exposure of ACR and calpeptin (a calpain inhibitor) prevented neurite degeneration, a classical effect of ACR \textit{in vitro}. ACR has also been shown to increase the levels of ROS. A reason for the increased levels of ROS could be that ACR exposure decreases the levels of neural glutathione and hence, attenuates the major cellular redox defense system.

As mentioned above, ACR reacts rather unspecific with sulfhydryl groups of cysteine residues in soft nucleophiles, making it difficult to understand why ACR induced toxicity is seen mostly at nerve terminals. The sulfhydryl group of cysteine can exist in various oxidative states. The anion state (-S\(^{-}\)) has been shown to be the major target of ACR. The equilibrium between the thiol (-SH) and thiolate (-S\(^{-}\)) states will shift towards the thiol state intracellularly since the pKa of cysteine is around 8.3 and the pH in the cytoplasm of the cell is around 7.4. However, there is evidence that specific cysteine residues have highly reactive thiolate moieties. These cysteine residues are components of catalytic triads that regulate different biochemical functions such as enzyme activities, membrane transporters, exchangers, and ion channels. These highly reactive thiolate moieties are effector sites for cellular redox-signaling pathways, including nitric oxide (NO) and hydrogen peroxide (H\(_2\)O\(_2\)). The modification of protein thiolate residues has been shown to modulate many pre- and postsynaptic features of neurotransmission. Taken together, one could hypothesize that neurotoxic compounds such as ACR could irreversibly modify sulfhydryl groups on specific cysteine residues, leading to disruption of redox-signaling pathways and sequential synaptic toxicity.
Developmental neurotoxicity of ACR
We are exposed to ACR throughout our lifespan, starting as early as during pregnancy. It has been shown that ACR crosses the placenta both in vitro and in vivo. Sörgel et al., demonstrated that ~20% of ACR could cross the human placenta and that ACR also crosses over into breast milk. The developing brain tends to be more sensitive to toxic stimuli due to many reasons such as the lack of a fully developed BBB or blood-cerebrospinal fluid barrier. At the same time, crucial initiating events are taking place in a strict and controlled order, leaving any alteration of this order, or the fine-tuning of it, to have possible detrimental effects on brain development. After birth, the exposure of ACR will continue through intake of food and drinks, but also from the environment. In the WHO report from 2005, it was estimated that the average consumption of ACR through food and drinks was 0.8-3 μg ACR/kg/day. However, the consumption in children was estimated to be 2–3 times higher than the adult consumption when expressed as a body weight ratio. Aside from the fact that children eat more food relative to their size, a lot of foodstuff containing high levels of ACR, e.g. cereal, French fries and potato chips, are preferred by children.

Even though ACR crosses the placental barrier, there are few studies on the effects of ACR on the developing nervous system. Research has shown that prenatal and perinatal exposure of ACR decreases the average horizontal motor activity and auditory startle response in exposed rats and that ACR impaired hippocampal neurogenesis in mice. In a study from 2018, it was shown that ACR produced learning and memory impairment in mice and they also showed that ACR exposure during the development of cultured primary neurons attenuated neuronal maturation without affecting viability. It has also been shown that ACR disrupts the development of cerebellum and that ACR induces lipid peroxidation and oxidative stress in the developing medulla oblongata in rats. Interestingly, neurodegenerative disorders are sometimes believed to be neurodevelopmental diseases to start with. Many neurodegenerative diseases share a common pathophysiological cascade involving oxidative stress, lipid peroxidation and the subsequent liberation of α,β-unsaturated carbonyl derivatives. The toxicity produced by these derivatives might be similar to the toxicity displayed by ACR. The derivatives will also form adducts with nucleophilic sulfhydryl groups on presynaptic proteins. The toxicity caused by the α,β-unsaturated carbonyl derivatives witnessed in neurodegenerative diseases and the neurotoxicity of ACR are both localized to the nerve terminals. ACR can react with nucleophilic scavengers, and hence, it has been suggested that the neuropathogenic process could be accelerated by ACR, leading to accelerated onset or progression of neurodegenerative diseases.

Methylmercury
MeHg is probably one of the most well-known and studied neurotoxic compounds. This is partly due to the many cases of accidental exposure that have taken place historically. One of the first evidences that MeHg exposure could lead to DNT occurred in the mid-1950s in Japan. Mothers who had eaten MeHg-contaminated fish from the Minimata Bay gave birth to children with different kinds of neurological defects while being totally unaffected or only mildly affected themselves. It was also clear that the damage inflicted by MeHg was different in the developing versus the adult nervous system. In the adult nervous system, the damage was restricted to the cerebellum and the visual cortex whilst the damage was more scattered and diffuse in the developing nervous system. The developing nervous system was also sensitive to far lower concentrations of MeHg. It was estimated that the developing nervous system had a five-fold increase in the sensitivity to MeHg compared to the adult nervous system. Children who have been exposed to MeHg during development in utero display various
different symptoms including intellectual disability, seizures, primitive reflexes and spastic paresis.\textsuperscript{384} The mechanistic reasons for these symptoms have been comprehensively studied throughout the years. When it comes to the half-life of MeHg, the existing studies are limited in their size and number, but from the available data, it appears that the $t_{1/2}$ for MeHg in plasma can vary in humans from 30 to 150 days.\textsuperscript{385} Obviously, the rate of elimination will influence the accumulation of MeHg and MeHg-induced toxicity.

The chemical properties of MeHg result in a very broad array of toxic outcomes. MeHg is a positively charged ion consisting of a methyl group (-CH$_3$) and a mercury ion (Hg$^+$). It is formed from pollution of inorganic mercury in the environment by methylation of microbes that live in water.\textsuperscript{386} MeHg can also be released into the atmosphere through the actions of volcano eruptions, forest fires or from mercury-containing rocks.\textsuperscript{387} Similar to ACR mentioned earlier, the positively charged ion of MeHg makes it form covalent bonds with anions such as chloride (Cl$^-$) and hydroxide (OH$^-$). It has high affinity for sulfur-containing anions such as the previously mentioned thiol group (-SH) of the amino acid cysteine. Since sulphydryl groups are common in proteins and often have relevance to the appropriate function of the protein, the formation of covalent bonds to MeHg at these locations can have detrimental consequences for the cell. Hence, many key functions in the cells are affected by MeHg including impaired proliferation, migration, growth cone activity, cytoskeletal functions, apoptosis and energy metabolism of cells.\textsuperscript{388} It has been shown that MeHg predominantly also binds to the cysteine group in the antioxidant glutathione, suggesting similar MOA for ACR and MeHg.\textsuperscript{389} Maintaining the levels of ROS has for example been shown to be crucial for regulation of self-renewal and differentiation of pluripotent cells.\textsuperscript{390} MeHg also passes through the BBB,\textsuperscript{388} the placental barrier\textsuperscript{391} and is excreted into breast milk.\textsuperscript{392}

Although we have massive knowledge about the association between DNT and MeHg exposure, there are still cases of poisoning happening. Multiple actions have been taken to reduce MeHg in the environment, e.g. banning the use of alkylmercury compounds as fungicides and constant monitoring of fish-contaminations. However, it seems like it is extremely difficult to get rid of any trace of the metal in fish and this might give rise to DNT in children with mothers that consume a lot of contaminated fish. There have been some contradicting results regarding low-level contaminated fish, and some studies have not found any relationship between the consumption and neurodevelopmental deficits in the offspring.\textsuperscript{393} One explanation for this might be that fish also contains beneficial components for a developing brain, such as omega-3 fatty acids. Hence, the harmful effects of MeHg would be balanced out by such components.\textsuperscript{394}

**Valproic acid**

VPA is a known DNT chemical and exposure during pregnancy has been associated with a number of developmental abnormalities including neural tube defects, spina bifida, autism and bipolar disorder.\textsuperscript{395} VPA a branched short-chain fatty acid that is derived from valeric acid that is a naturally occurring substance. VPA is used as a drug in the treatment of several different disorders such as epilepsy, seizures, migraine, bipolar disorder and anxiety.\textsuperscript{396} Most of the VPA is bound to proteins and the $t_{1/2}$ in plasma is around 18 hours for humans.\textsuperscript{397} VPA is metabolized in three ways, where the two major routes in humans is by glucuronidation and $\beta$ oxidation in the mitochondria, representing 50% and 40%, respectively. The remaining 10% is metabolized
The exact mechanisms of the pharmacodynamic effects of VPA are not fully understood and they are not limited to one function. VPA increased GABA levels by inhibiting GABA transaminase and succinate semialdehyde dehydrogenase, which are involved in the degradation of GABA. VPA has also been shown to block voltage-gated sodium-, potassium-, and calcium channels. VPA also acts as an inhibitor of different HDACs, thereby disrupting normal regulation of gene expression. In vitro transcriptomic studies revealed that VPA downregulated several genes involved in neural differentiation at the same time as upregulating genes involved in neural precursor proliferation.

**Nicotine**

Nicotine is a neurotoxin that is naturally produced in the plant *Nicotiana tabacum*. The neurotoxin has been used as an insecticide since the 1690s and has a lethal dose of 30-60 mg in humans. Signs of intoxication include nausea, vomiting, hypersalivation, increased pulse, increased blood pressure, bradycardia, tachycardia, dyspnea, convulsions, coma and respiratory arrest due to overstimulation of both the PNS and CNS. Nicotine is readily distributed in the bloodstream and can also cross the BBB. It is efficiently metabolized by CYP-mediated enzymes and has a t1/2 in plasma of 1-2 hours in humans. The mechanism for the neurotoxicity of nicotine is that it acts as an agonist for the nAChR. Nicotine has also been shown to induce DNT due to disruption of the nicotinic acetylcholine systems as well as a variety of neural circuits, e.g. the sensory motor system and emotional and cognitive functions.

**Atropine**

Atropine is naturally produced by the plant *Atropa belladonna*. The plant actually contains three tropane alkaloids: atropine, scopolamine and hyoscyamine. Atropine has a fatal dose of 15 mg in humans and signs of intoxication include dry mouth, urinary retention, flushing, papillary dilation, constipation, confusion and delirium – all symptoms caused by impairment of parasympathetic signaling. Atropine is readily distributed in the bloodstream and can also cross the BBB. It is efficiently metabolized and has a t1/2 in plasma of 1-2 hours in humans. The mechanism of the neurotoxicity of atropine is as an antagonist to the mAChRs.

**Strychnine**

Strychnine is a crystalline alkaloid neurotoxin found in the seeds of the *Strychnos nux-vomica* tree. The toxin has been used as a pesticide for many years, with the main target being small vertebrates such as birds and rodents. The fatal dose in humans is 1.5 mg/kg and intoxication leads to convulsions by muscular spasms and death through asphyxia. Strychnine has a t1/2 in plasma of 10 hours in humans. Strychnine acts as a competitive antagonist of glycine receptors as well as an antagonist for different acetylcholine receptors, this will generate an over excitation of inhibitory interneurons that will result in convulsions, muscle spasms and an exaggerated startle response.

**Digoxin**

Digoxin was first isolated in 1930 from the plant *Digitalis lanata*. It has been used as a drug to treat various heart conditions such as atrial fibrillation and heart failure. Digoxin is not reported to be neurotoxic and the main MOA of digoxin is cardiotonic with a t1/2 in plasma of 36 hours.
in humans. Digoxin increases the intracellular free Ca\(^{2+}\) concentration in cardiac myocytes as a result of Na\(^+/K^+\)-ATPase inhibition.\(^{413}\) Digoxin was used in paper IV as a non-neurotoxic control compound.

**Ethanol**

Ethanol is a well-known sedative as well as a DNT producing compound. In the CNS, ethanol acts by at least three MOAs, i.e. as a GABA\(_A\) receptor agonist (resulting in enhanced Cl\(^-\) influx), as a glutamate NMDA receptor antagonist (preventing postsynaptic excitatory potential) and by altering cell membrane fluidity. The overall effect of ethanol is attenuated excitability of neurons and sedation of the CNS, resulting in death if the dose gets too high.\(^{414}\)

**Acetylsalicylic acid**

Acetylsalicylic acid is a non-steroid, anti-inflammatory drug used to suppress pain, fever and inflammation. It is not classified as a neurotoxic or DNT producing chemical. However, it is not recommended to use high doses of acetylsalicylic acid during pregnancy unless it is necessary. Depending on the stage of the pregnancy acetylsalicylic acid can generate different risks. During the first trimester, use of higher doses creates a concern for pregnancy loss and congenital defects. During the third trimester, acetylsalicylic acid increases the risk of the premature closure of a vessel in the heart.\(^{415}\) These risks do not seem to be focused on the nervous system in particular. Acetylsalicylic acid suppresses the production of prostaglandins (pain inducing hormone) and thromboxanes (involved in platelet aggregation) by irreversible inactivation of cyclooxygenase 1 and 2.\(^{416}\) Acetylsalicylic acid was used in paper IV as a non-neurotoxic control compound.
Hypothesis and aim

This thesis is aimed at summarizing some of the alternative in vitro methods and models that have been used to study adult and developmental neurotoxicity, and also to pinpoint some of the important aspects of using alternative in vitro methods.

The aim of the papers included in this thesis was to challenge the hypothesis that acute and developmental neurotoxicity of chemicals can be studied using robust endpoints for proliferation and neural differentiation, e.g. neurite outgrowth, mRNA and protein expression. This was performed in two different cell lines: the murine neural progenitor cell line C17.2 and the human neuroblastoma cell line SH-SY5Y. We also sought out to characterize the two cell lines and identify marker genes important for differentiation. The objective being to evaluate if the markers could be used for investigation of DNT with the hypothesis being that any chemical that changes the expression of developmentally important genes could possibly result in DNT to the cells.

In this thesis, I have tried to answer some fundamental questions in the fields of acute neurotoxicology and DNT:
- Is it possible to predict human acute neurotoxicity and DNT in cell models without sacrificing any animals?
- Can whole genome mRNA analysis during differentiation be a valuable tool for selecting important markers for neuronal differentiation?
- Can mRNA expression of specific genes be used as markers for acute and developmental neurotoxicity?
- Are there correlations between gene expression and functional endpoints?
- Could the C17.2 cell line be as good as primary embryonic rat brain cell cultures for prediction of acute neurotoxicity?

Acrylamide was used as a model compound to answer these fundamental questions. It has been suggested that the neurotoxic compound ACR might also induce DNT. The goal was to investigate the mechanisms of ACR-induced DNT in the two cell models by studying robust endpoint such as the ones mentioned above. Should ACR intake and exposure during pregnancy be restricted, or does the DNT appear at concentrations higher than what is physiologically relevant?
Methodological considerations

Cell lines and cell culture
Two different cell lines were used in this thesis.

Neuroblastoma cell line SH-SY5Y
The human neuroblastoma cell line SH-SY5Y is a common cell line used as a model for studying neuronal function and differentiation.\textsuperscript{417,418} The SH-SY5Y cell line has been used extensively to look at DNT aspects in the past and was therefore chosen as a model cell line.\textsuperscript{125,419,420} The SH-SY5Y cell line is a third generation clone that originates from the cell line SK-N-SH. The SK-N-SH cell line was isolated from a neuroblastoma tumor in the bone marrow of a four-year-old girl in 1971.\textsuperscript{421} The SH-SY5Y cell line can be differentiated with various agents to a more neuronal-like phenotype. Undifferentiated SH-SY5Y cells are considered to have similarities with immature catecholaminergic neurons.\textsuperscript{422} Following treatment with differentiation-inducing agents, the cells become morphologically more similar to primary neurons with long, exquisite processes. Depending on the differentiation protocol, the differentiated cells may form many randomly scattered processes or they might become distinctly polarized. During differentiation, the SH-SY5Y cells decrease their proliferation rate and withdraw from the cell cycle. At the same time, there is an increased expression of the cyclin-dependent kinase inhibitors p21 and p27 and the anti-apoptotic proteins Bcl-2 and Bcl-xL. There is also an increased activity of the PI3K/AKT pathway, which plays a role in neurite development and differentiation, and an increase of neuronal markers such as neuron specific endolase.\textsuperscript{423} RA is the most commonly used differentiation-inducing agent to study neurite outgrowth in SH-SY5Y cells.\textsuperscript{424} Differentiation with 1 µM RA was used for all experiments with SH-SY5Y cells in this thesis, which according to published literature produces a culture with a cholinergic-like phenotype and might increase the susceptibility of the cells to neurotoxins and neuroprotective agents.\textsuperscript{422,425} Differentiation of SH-SY5Y with RA in combination with other agents, such as phorbol esters, can generate a more mature dopaminergic phenotype and decreases the susceptibility of cells to neurotoxins and neuroprotective agents.\textsuperscript{426} Differentiation with 12-0-tetradecanoyl-phorbol-13-acetate alone has been documented to generate a more adrenergic phenotype.\textsuperscript{424}

For routine cultures, the SH-SY5Y cells were cultured in MEM supplemented with 10% fetal calf serum, 1% MEM non-essential amino acids, 2 mM L-glutamine, 100 µg streptomycin/mL, 100 U penicillin/mL. The confluent cells were detached once a week using 0.05/0.02% trypsin/EDTA and seeded in a new cell culture flask at the original density of $27 \times 10^5$ cells/cm$^2$. For differentiation studies, the SH-SY5Y cells were seeded in routine culture medium. Twenty-four hours after seeding the medium was changed to DMEM/F-12 medium supplemented with 1 mM L-glutamine, 100 U penicillin/mL, 100 µg streptomycin/mL, N2 supplements (0.9 mM bovine insulin, 20 nM progesterone, 30 nM sodium selenite, 100 ng/mL bovine apo-transferrin, and 100 µM putrescine dihydrochloride),\textsuperscript{427} and 1 µM of RA. The differentiation medium was changed every third day for the duration of the differentiation.
Neural progenitor cell line C17.2

The C17.2 cell line was originally cloned from mouse cerebellar NPCs and was immortalized through v-myc retroviral transduction.\(^{428}\) The cells were isolated on postnatal day 4 from a male mouse from the K-strain.\(^{429}\) This cell line was a generous gift from Professor Sandra Ceccatelli (Karolinska Institute, Stockholm, Sweden) with permission of Professor Evan Snyder (Harvard Medical School, Boston, USA). The C17.2 cell line is a multipotent progenitor cell line that can differentiate into neurons and glia cells as a mixed cell population by the addition of NGF and BDNF to serum-free DMEM/F12 medium with N2 supplements.\(^{430}\) The C17.2 cell line has also been used for DNT testing in the past\(^{431-433}\) and was chosen due to its ability to differentiate into a mixed co-culture of neurons and glia cells, as well as for being very easy to handle.

For routine cultures, the C17.2 cells were cultured in DMEM supplemented with 5% horse serum, 10% fetal calf serum, 2 mM L-glutamine, 100 U penicillin/ml and 100 µg streptomycin/ml. The confluent cells were detached every third and a half day using 0.05/0.02% trypsin/EDTA and seeded in a new cell culture Petri dish at the original density of 1.27 x 10\(^3\) cells/cm\(^2\). For differentiation studies, the C17.2 cells were seeded in routine culture medium. Twenty-four hours after seeding the medium was changed to DMEM/F-12 medium supplemented with 1 mM L-glutamine, 100 U penicillin/mL, 100 µg streptomycin/mL, N2 supplements (0.9 mM bovine insulin, 20 nM progesterone, 30 nM sodium selenite, 100 ng/mL bovine apo-transferrin, and 100 µM putrescine dihydrochloride),\(^{427}\) 10 ng/mL NGF and 10 ng/mL BDNF. The differentiation medium was changed every third day for the duration of the differentiation.

Cell exposure

The chemicals were dissolved in the cell culture medium used for the different experiments. Before being applied to the cells, the stock solutions were sterile filtered through a 0.2 µm syringe filter and then diluted to the desired concentrations. Cell cultures exposed to medium without the chemical in question were used as a control. For paper I-III, exposure started 24 hours after seeding, concomitantly with the change of the culture medium, i.e. at the change to differentiation medium for the differentiation studies and change to fresh routine culture medium for proliferation studies. The chemicals were added at every change of medium throughout the duration of the experiment. Fresh medium without the chemical was added to control cells. A fresh stock solution of the chemical was prepared right before addition to the cells for each exposure time. The exposure time and concentrations differed between the two cell lines. See individual experiments in the articles for more detailed information.

MTT assay

The MTT assay is a tetrazolium based metabolic cell proliferation assay. It was first described by Mosman and coworkers in 1983 as the first viability assay that was designed for 96-well plates to be used for high-throughput screening.\(^79\) The method is based on the concept that MTT is added to the cell culture and taken up into the cells. Once MTT is inside the cells, it can be converted to the purple colored formazan. The concept being that only viable cells have ability to reduce MTT to formazan. The exact mechanism of formazan production is debated, but the common assumption is that MTT probably reacts with NAPD or other reducing molecules that transfers electrons to MTT.\(^{434,435}\) There were early publications stating that MTT reduction was
due to the activity of enzymes specific for the mitochondria.\textsuperscript{436} This lead to publications referring to the MTT assay as a measurement of mitochondrial activity. However, it has since then been shown that this is not the only sight of MTT reduction. MTT reduction also occurs in the cytoplasm and in association with membranes in the endosome/lysosome compartment as well as at the plasma membrane.\textsuperscript{437} The purple formazan product is not water soluble and the precipitate will accumulate inside the cells. The crystals can be dissolved with various solvents such as dimethylsulfoxide, dimethylformamide or sodium dodecyl sulfate. If sensitivity is a problem, it might be good to acidify the solvent first, as it turns the phenol red to yellow and that will reduce the interference with the absorbance readings.\textsuperscript{438} Once the crystals are dissolved, the absorbance is measured in a spectrophotometer, usually at 500-600 nm wavelength. The readout from the measurement can be dependent on many parameters such as the MTT concentration added to the cells, the duration of the MTT incubation, the total amount of viable cells in the culture, how metabolically active the cells are and the wavelength chosen.\textsuperscript{438} Since all of these parameters can affect the results, they are important to consider when optimizing the assay for the specific experiment being performed. For all MTT assays performed in this thesis, the protocol was optimized by using different seeding cell densities, MTT concentrations and wavelengths for absorbance measurement.

**Resazurin based assays**

Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a blue dye that has been widely used to measure cell viability in cytotoxicity assays. The dye was first used in 1929 to measure bacterial content in milk.\textsuperscript{439} Its low toxicity compared to the MTT assay has made it a more suitable choice when studying cell viability. The method was first put on the market commercially as AlamarBlue\textsuperscript{®} but is now sold under many different names, e.g. Vybrant and UptiBlue. The resazurin assays are based on the irreversible enzymatic reduction of resazurin to the highly fluorescent pink reagent resorufin. The conversion of resazurin to resorufin is proportional to the metabolic activity of a cell culture.\textsuperscript{440} Resorufin fluorescence can be measured at excitation 540 nm and at emission 590 nm with a fluorometer accommodated for multi-well plates.

**Lowry assay**

The Lowry assay is copper-based protein assay.\textsuperscript{441} The exact mechanism behind the formation of the measured product is not known. However, the concept is based on the fact that peptides containing two or more amino acid residues, under alkaline conditions containing sodium potassium tartrate, form a complex with divalent copper ions. The complex consists of 4 peptide bonds and one atom of copper. It is thought that this complex can reduce the Folin reagent added to produce a colored product. This product is light blue to violet and can be measured in a spectrophotometer at a wavelength between 650 and 750 nm. The intensity of the color is proportional to the number of peptide bonds created, hence gives a measurement for protein concentration. The absorbance is measured against a standard curve of a selected standard protein solution, e.g. bovine serum albumin. The amount of certain amino acid residues in the peptide backbone (tyrosine, tryptophan, cysteine, histidine, asparagine) enhances the color of the assay since the radical groups of these residues further reduce the Folin reagent.\textsuperscript{442} The Lowry assay should always be run in a minimum of duplicates,\textsuperscript{443} in paper I the assay is run in triplicates. One should also keep in mind that agents such as commonly used detergents,
reducing agents and buffers can interfere with Lowry protein determination.\textsuperscript{444} The Lowry assay is extremely temperature, pH and time sensitive and it is important that the incubation times and the procedures are reenacted the same way when the assay is repeated. There can also be a problem if you have too high protein concentrations due to saturation of the absorbance.

**Bradford assay**

The Bradford assay is an assay for measuring total protein concentration by using Coomassie G-250 dye in a colorimetric reagent.\textsuperscript{445} The protein in the sample will bind to the Coomassie dye under the acidic conditions provided by the reagent. This will create a spectral shift in the dye. This shift is preferably measured at the wavelength where the difference between the two dye forms is the greatest, i.e. at 595 nm. The absorbance is measured against a standard curve of a selected standard protein solution, e.g. bovine serum albumin. There are certain considerations regarding the Bradford assay that should be considered. The development of the shift in color is associated with the existence of certain basic amino acids within the protein (mostly histidine, lysine and arginine). Also, the number of Coomassie dye ligands bound to a protein is proportional to the amount of positive charges in the protein. The assay cannot detect free amino acids nor peptides or proteins smaller than 3000 Daltons. Similar as with the Lowry assay, there can also be a problem if you have too high protein concentrations due to saturation of the absorbance.

**Proliferation assays**

As mentioned in the introduction, proliferation can be studied in several different ways. Two different proliferation assays were used in this thesis and will be covered here in more detail. The thymidine incorporation assay measures the incorporation of a radioactively labeled nucleoside thymidine into new strands of chromosomal DNA. The amount of incorporated $^3$H-thymidine is measured with a scintillation beta-counter.\textsuperscript{446} Upon detecting incident radiation, the scintillator generates photons that are converted to an electrical signal that can be detected by the machine as disintegrations/minute. In this way, the amount of signal that you get can be correlated to the amount of proliferation in your sample. The same strategy is used in the BrdUrd assay. However, instead of using a radioactive nucleotide, the BrdUrd assay uses the nucleoside analog 5-bromo-2’-deoxyuridine that is detected using antibody probes for BrdUrd with for example immunohistochemistry or like in paper I, by flow cytometry.\textsuperscript{447}

**Cell cycle analysis**

One of the earliest applications for flow cytometry analysis was to quantify the amount of DNA.\textsuperscript{448} There are many DNA staining dyes that can be used for mammalian cells, e.g. PI, Hoechst 33342 or DRAQ5. These dyes are stoichiometric, which means that they will bind proportionally to the amount of DNA that is present in the cell. PI is probably the most common dye to use when studying the cell cycle. PI is added to permeabilized cells and binds to DNA with a stoichiometry of one dye per 4-5 base pairs of DNA. The dye is a DNA intercalating dye that has little or no sequence preference and therefore it also binds to RNA. To eliminate RNA binding the samples have to be treated with RNase before measuring the DNA content. Once the dye is bound, the fluorescence excitation maximum is shifted to the red spectrum and the fluorescence emission maximum is shifted to the blue spectrum, which makes the fluorescence intensity detectable by for example flow cytometry or fluorescence microscopy. The different cell cycle phases can be seen in Figure 8A. Briefly, the period between mitotic divisions, i.e.
the G1-, S- and G2 phase, is known as interphase. During the G1 phase the cell undergoes metabolic changes and prepare for cell division by e.g. copying organelles. At a certain point called the restriction point, the cell is committed to division and moves into the S phase. During S phase DNA is replicated and each chromosome will by the end of the S phase consist of two sister chromatids. During the G2 phase metabolic changes occur to assemble the cytoplasmic materials necessary for mitosis and cytokinesis. The G2 phase is followed by the M phase (mitosis) where a nuclear division takes place followed by a cell division (cytokinesis). Since the dyes used for cell cycle analysis are stoichiometric, the cells in the S phase will display increasing fluorescence intensity up until all of the genetic material is replicated. Once all of the genetic material is replicated the cells will enter the G2 phase and will display twice the amount of fluorescence as the cells in the G1 phase. Cells in the G2 phase and mitosis cannot be distinguished since they both display the same amount of DNA (Figure 8B).

![Cell cycle phases](image)

**Figure 8.** A) The different cell cycle phases during mitosis. B) DNA histogram displaying the different cell cycle phases analyzed by flow cytometry after DNA staining. Image adapted with permission from (Tabll and Ismail, 2011).

**Multiplex neurite branching-PI-ATP assay**

This assay can be used to study the effect of a chemical on neurite outgrowth, cytotoxicity and ATP content. High content imaging is used for measuring the neurite length after staining the cells with the permeant dye calcein-AM. Calcein-AM is hydrolyzed after entering the cell, producing a fluorescent signal, which allows the visualization of the cellular cytoplasm of live cells. The correlation between cell bodies and nuclei is determined by using Hoechst 33342 as nuclear staining. Differentiation between live and dead cells is at the same time achieved with PI staining. The cells are imaged with an automated imaging system (ImageXpress® Micro, Molecular Devices) and image acquisition software MetaXpress® (Molecular Devices) using a modified version of the neurite outgrowth application module. Different wavelengths were used for detection of the different dyes, i.e. Hoechst (excitation 377/50 nm, emission 447/60 nm and 100 ms exposure time), calcein (excitation 482/35 nm, emission 536/40 nm and 120 ms exposure time) and PI (excitation 562/40 nm, emission 624/40 nm and 400 ms exposure time). After imaging fluorescence of calcein, Hoechst and PI, the ATP content is measured with
CellTiter-Glo® 2.0 (Promega) in the identical cells. The ATP assay uses a thermostable luciferase, which produces a luminescent signal proportional to the amount of ATP present in the analyzed samples.

Quantification of the mean neurite branching was determined with the neurite outgrowth standard algorithm for MetaXpress® software (Molecular Devices). Cells stained with Hoechst and calcein were considered as live cells. For live cells, the length of the branches of the neurites (process branching) was quantified and the mean branching of each concentration was normalized against the mean neurite branching of the negative control (i.e. untreated cells). For the cytotoxicity analysis, the total number of imaged cells was calculated by combining the number of cells stained with PI to the number of live cells stained with calcein. In the ATP content assay, the average luminescence value of each concentration was normalized against the mean luminescence value of control wells and subsequently multiplied by 100 in order to obtain the % of viable cells in each concentration.

Quantitative reverse transcription polymerase chain reaction
Quantitative reverse transcription polymerase chain reaction (RT-qPCR) is a highly sensitive technique for detection and quantification of RNA. The technique is based upon two steps. The first step is reverse transcription synthesis of complementary DNA (cDNA) from RNA. Reverse transcriptase is an RNA-dependent DNA polymerase. In this step, first-strand cDNA is created using a poly-A oligonucleotide as a primer. This works for all prokaryotic and eukaryotic mRNA since they have a poly-A tail at their 3'-ends. The primer anneals to the 3'-end of the mRNA and is extended with the help of reverse transcriptase, creating an RNA-DNA hybrid. This molecule can be used as a template for the real-time qPCR, or the RNA strand can first be removed by RNase treatment. Once cDNA is formed, the second step of the RT-qPCR procedure begins. The second step is the amplification of specific cDNA by the PCR. The basic principle of the PCR is that selected primers for the gene-coding sequence of interest in the cDNA is added together with DNA polymerase. This procedure consists of 3 steps that are repeated in cycles to amplify the product: (i) denaturation (ii) annealing (iii) extension. The selected cDNA is amplified exponentially and this can be monitored quantitatively in real time using different detection methods. One common detection method is using non-specific fluorescent dyes that intercalate in to all double-stranded DNA. Another common method is using sequence-specific DNA probes that are fluorescently labelled to a reporter that will allow detection after the probe hybridizes with its complementary sequence. The thermal cycler reads the signal after each cycle and detects the intensity. For this thesis, SYBR green was used as a non-specific fluorescent dye. The cycle threshold (Ct) is set to be the cycle where the fluorescent detection crosses the threshold, i.e. exceeds the background level. All cDNA products will reach the threshold after varying number of cycles, depending on the initial amount of the cDNA in the sample. Hence, the Ct-values are inversely proportional to the amount of target nucleic acid in the sample. The individual Ct-values for the target genes are normalized against one or more reference genes. The choice of reference genes is very important since it will have a huge impact on the results. Reference genes should be selected carefully and should fulfill certain criteria. First of all, the reference genes should be equally expressed during all stages of differentiation relative to the total amount of cellular mRNA. They should also have a strong and stable expression.
Western Blot
Western blot has since its discovery been a commonly used method to quantify the amount of a specific protein in a sample.\textsuperscript{456} The method is based on the following principles. Sodium dodecyl sulfate and the sample are boiled together to denature the proteins. This procedure also gives the proteins a negative net charge, which is used to separate the proteins relative to their size in a polyACR gel when run in an electric field. After the proteins have been separated in the gel, they are transferred with the help of an electric current to a nitrocellulose or polyvinylidene difluoride membrane. Once the proteins are transferred, the membranes can be probed using specific antibodies to the proteins of interest. The primary antibodies are then detected using a secondary antibody conjugated with e.g. horseradish peroxidase, which makes it is possible to visually detect the protein by enhanced chemiluminescence. This is accomplished by adding a solution consisting of hydrogen peroxide and luminol to the membrane. The horseradish peroxidase will catalyze the oxidation of luminol by hydrogen peroxide, which creates acridium ester intermediates. These intermediates react with peroxide and generate an excited state, which during its decline to a lower energy level will emit luminescence that can be detected as specific bands and there after quantified by densitometric analysis.

Immunofluorescence microscopy
Immunofluorescence microscopy is a powerful analytical tool that combines the magnifying properties of light microscopy with visualization of fluorescence. This method is based on pioneering work done by Coons and Kaplan.\textsuperscript{457} Basically, immunofluorescence microscopy is a way to visualize cellular proteins by antibodies conjugated to a fluorescent dye, either directly through the primary antibody or indirectly through a secondary antibody. Indirect immunofluorescence is more commonly used as it generates higher sensitivity. The fluorescent signal is amplified since more than one secondary antibody can bind to each primary antibody. Indirect immunofluorescence also tends to be more flexible and cheaper.

In-depth gene expression analysis – whole genome microarray and RNA-sequencing
When it comes to whole genome in-depth gene expression analysis there are multiple methods to choose from. The use of microarray analysis was very popular about a decade ago, but has since then been gradually replaced by sequencing based analyses. A microarray consists of huge amount of oligonucleotides with known sequences that are located on a specific location on a chip. After RNA extraction of your sample, the RNA is reverse-transcribed and subsequently labeled so that hybridized cDNA can be detected and quantified.\textsuperscript{458} As mentioned, this method has during recent years been replaced to a large extent by next-generation sequencing techniques such as RNA sequencing (RNA-seq). This is partly because RNA-seq has become more affordable (however it is still more expensive than microarrays) with the development of more efficient methods but also because of its many advantages. Some of the advantages with RNA-seq compared to microarrays are that it is possible to study a broader dynamic range, i.e. RNA-seq is not limited by background at the low end and signal saturation at the high end. Furthermore, it is possible to study variations in DNA (e.g. single nucleotide polymorphisms, insertions, deletions), and it is also possible to discover alternative splice variants and
previously unknown genes since there is no need for species- or transcript-specific probes. RNA-seq is also more sensitive and has higher specificity resulting in a higher detection of differentially expressed genes, in particular genes with lower expression.459

For the RNA-seq, the quality of the sequencing data was analyzed using the Fastqc package (version 0.10.1) and after analyses the tails of the sequences were trimmed of the bad length (12 nucleotides) using Trimmomatic (version 0.33). Mapping of the reads on the human genome was done using Bowtie2 (version 2.2.6) and calculating the read counts was done with RSEM (version 1.2.28). For the microarray assay, the raw data was normalized in the free software Expression Console provided by Affymetrix (http://www.affymetrix.com) using the robust multi-array average (RMA) method first suggested by Li and Wong.460

Biostatistical comparison between the treated samples and the untreated control/undifferentiated samples can be done in many ways. Most of the different methods can be found as packages in the Bioconductor project.461 In this thesis, we used the limma package for Paper II and the DESeq2 package for paper III. The DESeq2 package uses counts as input data and a negative binomial approach, whereas limma uses a linear model for which the count data needs to be transformed to continuous values.463 Principal component analysis plots of the rlog transformed count data were generated as part of the DESeq2 workflow. Genes with a false discovery rate (FDA) adjusted p-value < 0.05 and absolute log2(fold change) > 1 were considered as differentially expressed. Venn diagrams of the differentially expressed genes (DEGs) between the three contrasts were generated using a web tool developed by the Bioinformatics & Evolutionary Genomics Laboratory at VIB/UGent, Belgium (http://bioinformatics.psb.ugent.be/webtools/Venn/). Canonical pathway analysis was performed on the DEGs using Ingenuity Pathway Analysis (IPA) software (license obtained from Ingenuity Systems, Redwood City, CA). In paper II, the analysis-ready genes were used for canonical pathway analysis as well as disease and function analysis. Output data were used to generate heatmaps of the top 20 enriched pathways according to the p-value as well as z-score (measure of pathway activation/inhibition). To select relevant markers for neural differentiation of the C17.2 cell line, gene set enrichment analysis (GSEA) was performed on the genes selected as differentially expressed (absolute log2(fold change) > 1, p-value < 0.05) and from the genes that were determined as differentially expressed, all genes involved in gene sets connected to the brain and neural functions were further selected. From this list of differentially expressed genes involved in neural differentiation, the 30 genes with the highest log2(fold change) changes were chosen without bias. For paper III, the canonical pathway analysis was also performed on the DEGs using IPA software (license obtained from Ingenuity Systems, Redwood City, CA). The enriched pathways were narrowed down to the ones part of the ‘Neurotransmitters and other nervous system signaling’ and ‘Ingenuity toxicity pathways’ category. Heatmaps of the genes differentially expressed within the CREB and RAR activation pathways were generated using data output from IPA and the pheatmap Bioconductor package.

Cell membrane potential
There are multiple ways of measuring the CMP. Neher and Sakmann got the Nobel Prize in 1991 because of their work during the 1970s and early 1980s in regards to measuring CMP with the patch clamp technique.464 This technique uses electrodes and allowed electrophysiological
recordings of single ion channels for the first time, which contributed to our understanding of the specific role of different ion channels in action potentials and nerve function in general. It is also possible to measure changes in membrane potential by using molecules that change their physicochemical features depending on the electrical field that surrounds them. Molecules like these are usually good for measuring fast responses and can measure transient (millisecond) CMP changes in excitable cells. There are also slow-response probes using anionic dyes for detection of CMP changes. Due to the charge of the dye, it can only enter the cells during depolarization. Inside of the cells, the dye binds to intracellular proteins and emits a fluorescent signal with a different shift. Hence, increased depolarization of the cell membrane generates an increase in fluorescence and hyperpolarization of the cell membrane generates a decrease in fluorescence. In paper IV we used a probe called FLIPR membrane potential (FMP) Red probe. The FMP probe is a slow-response probe but has been shown to be 14-fold faster than traditional slow-response probes such as DiBAC dyes. During depolarization of the cell membrane, the FMP probe translocates into the cell where it binds to hydrophobic sites on the intracellular side of the plasma membrane, resulting in an increase in fluorescence intensity. The opposite happens during hyperpolarization, where the probe is forced out from the cell by the negative electrochemical gradient, resulting in a decrease in the fluorescence signal. The cells were cultured in 96-well plates and read in a semi-HTS-fluorescence plate reader (the Flexstation II from Molecular Devices). Like with most assays, there are pros and cons. Some limitations with the FMP probes are for example that the full structure and function of the probes is unavailable and it is therefore difficult to predict if some chemicals may interact with the probe and influence the outcome of the assay. Quenching of chemicals with the probe will result in reduced fluorescence due to absorption of emitted light. There might also be autofluorescence and hence it is important to determine the correct background before performing the calculations.
Results and discussion

Paper I – ACR affects proliferation and differentiation of the neural progenitor cell line C17.2 and the neuroblastoma cell line SH-SY5Y

The aim of this paper was to identify modes of action for ACR induced developmental neurotoxicity. To this end we tested two different cell lines, i.e. the murine neural progenitor cell line C17.2 and the human neuroblastoma cell line SH-SY5Y. We evaluated endpoints such as proliferation of progenitor cells before and during differentiation, neurite outgrowth and mRNA as well as protein markers for neural differentiation.

During the initial phase of brain development there is an expansion of neural progenitor cells. Studying the proliferation of undifferentiated neuronal and glial precursors can be used as a model to mimic the expansion of progenitor cells and hence, the proliferative stage of neural development. We studied how ACR affected precursor proliferation and cell death in the two cell lines by performing several different assays such as growth curves by cell counting, BrdUrd incorporation, MTT cell viability assay and we also studied the cell cycle phases by flow cytometry. By combining an assay for cell cycle phase distribution (including the Sub-G1 phase reflecting cell death) with a proliferation assay, it was possible to determine if the decreased viability seen in the MTT and growth curve assays was due to reduced proliferation, cell death or a combination of both. In the MTT assay, we observed that the number of viable cells was significantly reduced in both cell lines after 3 days of exposure with 750 µM of ACR. Prolonged exposure to SH-SY5Y cells with ACR showed a significant reduction in the number of viable cells, already at 250 µM of ACR compared to control cells. The BrdUrd incorporation assay and the Sub-G1 phase of the DNA histogram showed that the reduction in MTT absorbance observed with increasing ACR concentrations was due to a combination of both reduced proliferation and increased cell death. However, the viability of the progenitor cells was affected at very high concentrations (> 250 µM), i.e. concentrations that are not physiologically relevant for the fetus during normal maternal exposure. With that said, it might still be that prolonged chronic exposure to lower doses of ACR could affect the proliferation of progenitor cells in the fetus, since we know that the toxic effects of ACR manifests in a time-dependent manner both in vitro and in vivo. These findings are important considering that they show that ACR might not only be toxic to differentiated neurons or astrocytes but also to their progenitor state. This indicates that ACR exposure might have implications very early on during pregnancy.

We moved on to study adverse effects of ACR on neural differentiation. We started off by studying the number of viable cells during differentiation and ACR exposure by the MTT assay. Both cell lines were exposed to a wide range of ACR concentrations throughout 6 and 10 days of differentiation of the C17.2 neural progenitor cells and for 3 and 6 days of differentiation of the neuroblastoma SH-SY5Y cells. The different durations of exposure were used to reflect different exposure windows during neurodevelopment (Figure 9). The undifferentiated C17.2 cells are neural progenitor cells and are hence more naïve than the undifferentiated neuroblasts.
Neural progenitor cells still display symmetric cell division and hence need longer differentiation to reach the same level of maturity as the SH-SY5Y cells.

Figure 9. Image of different processes during brain development. The image shows where the C.17.2 and the SH-SY5Y cell model fits in to the developmental timeline of the nervous system from the formation of the blastocyst to adulthood.

ACR significantly reduced the number of viable C17.2 cells in the MTT assay during differentiation in a time- and concentration-dependent manner, starting at 100 µM after 6 days of exposure and 10 µM after 10 days of exposure. This was in line with the cytotoxicity in undifferentiated cells. The SH-SY5Y cells initially displayed an increase in the number of viable cells during exposure to low concentrations of ACR (10 pM-10 µM) followed by a decrease at concentrations over 500 µM in comparison with differentiating control cells. The fact that viability declined at concentrations >500 µM was not surprising since ACR has been shown to be cytotoxic to differentiated SH-SY5Y cells around concentrations of 0.8-1 mM. To confirm that the increase in MTT reduction after exposure to low concentrations reflected the amount of viable cells rather than increased metabolic rate, the effect of ACR on the total cellular protein concentration was determined by the Lowry assay. The cellular protein concentrations followed the same trend as the MTT viability assay, manifesting that very low concentrations of ACR increased the number of cells and that higher concentrations (>500 µM) induced cytotoxicity and a decrease in cell number in comparison to unexposed control cells. We also performed the ³H-thymidine incorporation proliferation assay to see if the increase in MTT reduction after exposure to low concentrations reflected the amount of viable cells rather than increased metabolic rate, the effect of ACR on the total cellular protein concentration was determined by the Lowry assay. The cellular protein concentrations followed the same trend as the MTT viability assay, manifesting that very low concentrations of ACR increased the number of cells and that higher concentrations (>500 µM) induced cytotoxicity and a decrease in cell number in comparison to unexposed control cells. We also performed the ³H-thymidine incorporation proliferation assay to see if the increase in cell numbers correlated with an increase in cell proliferation. The ³H-thymidine incorporation assay is a very sensitive assay and could in fact detect a significant increase in cell proliferation at low exposure starting already at 10 fM both after 3 and 6 days of differentiation. The fact that ACR increased cell proliferation and the number of viable cells at low concentrations suggests that ACR might attenuate the differentiation process, keeping the cells in their mitotic neuroblast stage.

We also looked at the mRNA and protein levels of the neural markers βIII-tubulin and GFAP in the C17.2 cell line by RT-qPCR, western blot and immunofluorescence microscopy. βIII-tubulin is a marker for semi-mature neurons and it was selected as the marker for neuronal differentiation. GFAP is the most commonly used marker for astrocytes, hence it was used as indicator of astrocytes in the differentiated C17.2 cell cultures. In the C17.2 cells, both the mRNA and protein levels of βIII-tubulin and the GFAP were reduced after ACR exposure during differentiation. We also studied how ACR affected the ratio between the
different cell populations in the mixed co-cultures during exposure. ACR exposure decreased the number of neurite-bearing C17.2 cells. However, the number of neurites per cell was not affected by ACR and the fraction of non-neurite-bearing cells increased with ACR exposure. These findings, in combination with the decrease seen in the GFAP level, indicate that the cells might be less differentiated and hence, stay in their naïve progenitor state. The fact that both phenotypes are affected might result in even worse complications for the developing brain.

The SH-SY5Y cell line was also used to study neurite outgrowth during differentiation and ACR exposure. The number of neurites was significantly reduced after 6 days of exposure to 10 pM of ACR and by 100 nM after 3 days of exposure. Taken together, ACR impairs neurite outgrowth in the SH-SY5Y cell line during differentiation in a time- and concentration-dependent manner. Western blot was also used to study the expression of βIII-tubulin in SH-SY5Y cells during differentiation and ACR exposure. Although not significant, the protein levels of βIII-tubulin showed a slight decline during ACR exposure.

The differences in sensitivity to neurite outgrowth impairment between the two cell lines might be explained by the fact that the astrocytes in the differentiated, mixed C17.2 cell culture can buffer some of the neurotoxicity, e.g. by providing additional conjugation-sites for ACR such as glutathione.472 Astrocytes also have a pivotal role in the function and support of neurons, given the fact that they release factors that sustain neuronal function and viability. For instance, astrocytes synthesize and secrete a wide range of cytokines, neurotrophic factors, growth factors, proteoglycans, extracellular matrix proteins and cholesterol that are involved in neuronal proliferation, differentiation, synaptogenesis and survival.473,474 One should also mention that the two cell lines derive from two different species, i.e. the C17.2 cell line is murine and the SH-SY5Y cell line is human. It should not be excluded that some of the differences seen in the sensitivity to ACR exposure could be due to variances between the two species.

In conclusion, paper I shows that ACR affected the early phases of neurodevelopment, i.e. the expansion of neural progenitor cells and neuroblasts. ACR also affected the differentiation process by reducing markers for neural differentiation and axonal outgrowth. We show that ACR impaired neural differentiation at 7 orders of magnitude lower concentrations than previously reported in different neural cell systems.475–477 Our results show that ACR affected differentiation starting at 10 fM, which is 7 orders of magnitude lower than the estimated plasma concentration of free ACR in the fetus.475 The present study suggests that the risk for ACR-induced developmental neurotoxicity should be further evaluated and the recommendations of the tolerated daily intake of ACR during pregnancy and for children in general, may need to be reevaluated.

In addition, GFAP was chosen as a marker for astrocytes due to the fact that it is a well-known and accepted marker. However, looking at our results it is clear that both the neurons and the astrocytes stain positive for GFAP. It has been shown that there can be neuronal expression of GFAP, which is mostly associated with Alzheimer’s pathology.479 With that in mind, we recommend the use of an alternative astrocytic marker for future experiments.
The aim of paper II was to use transcriptomic microarray gene expression analysis in order to identify a panel of mRNA markers that were critical for differentiation of the neural C17.2 progenitor cells and evaluate if these selected mRNA markers could be used to alert for possible DNT inducing chemicals. From a whole genome Affymetrix micorarray analysis, we evaluated the gene expression of selected markers following exposure to four xenobiotics. Two known DNT-inducing compounds were used as positive controls, i.e. MeHg and VPA and the neurotoxic compound ACR was studied as a potentially DNT-inducing agent. D-mannitol was used as a negative control.

As mentioned in the introduction, next generation of DNT testing is envisioned to combine both in silico and in vitro testing methods in order to generate a more rapid and efficient toxicity screening. When it comes to in vitro studies, different endpoints such as neurite outgrowth and neural proliferation have been reported to be important for detection of DNT. In addition, several studies have identified mRNA markers of neural differentiation to be useful for toxicity screening. However, from a regulatory point of view, single endpoints/markers will simply not be sufficient for in vitro DNT testing. Testing strategies for DNT should be comprehensive, i.e. include a battery of relevant endpoints and should give mechanistic insight to the MOA, discriminate between different neural subpopulations as well as different stages of neural differentiation. International stakeholders have proposed a DNT testing strategy based on compound testing across a battery of in vitro tests, which include the important factors of timing and dynamic processes of brain development. The use of mRNA markers is a good example of such approach for DNT test systems. The relevance of toxicogenomic approaches in safety testing is widely recognized. According to a revision of non-clinical safety studies, it was concluded that significantly regulated transcripts might serve as robust markers of toxicity. The study showed that there was poor correlation with histopathological findings, however, transcriptomics showed to be a very sensitive marker for toxicity and often preceded more traditional endpoints.

The C17.2 cells were differentiated for 10 days and compared to undifferentiated cells. From the results of the whole genome microarray, 30 genes were selected from the differentially expressed genes that are involved in neural networks according to the Ingenuity Pathway Analysis software (IPA, content version 26127183, Ingenuity Systems, Redwood City, CA) as well as manual review of published literature. Briefly, the contrast Day 10 vs Day 5 had 307 differentially expressed (absolute log2(fold change) > 1, p-value < 0.05) probe set IDs out of which 285 were mapped to gene symbols (using IPA). After removing duplicates there were a total of 283 genes ready for analysis (192 upregulated, 91 downregulated). The analysis-ready genes were used for canonical pathway analysis as well as disease and function analysis. Output data were used to generate heatmaps of the top 20 enriched pathways according to the p-value as well as z-score (measure of pathway activation/inhibition). To select relevant markers for neural differentiation of the C17.2 cell line, gene set enrichment analysis (GSEA) was performed on the genes selected as differentially expressed (absolute log2(fold change) > 1, p-value < 0.05). From the genes that were determined as differentially expressed, all genes involved in gene sets connected to the brain and neural functions were further selected. From this list of differentially expressed genes involved in neural differentiation, the 30 genes with
the highest log2(fold change) changes were chosen. The mRNA markers that were selected were involved in most of the important features of neural differentiation such as neurogenesis, axonogenesis, axonal guidance, astrocyte- and oligodendrocyte differentiation and neuronal connectivity. By selecting genes that are involved in a broad perspective it further increases the chance of identifying substances with wide ranges in their MOAs. The reason for choosing a limited set of mRNA markers, instead of the whole genome, is that it makes this model affordable and high-throughput which is in the lines with the recommendations within the field as mentioned above. The 30 selected mRNA markers were validated using RT-qPCR.

To evaluate the importance of the mRNA markers chosen, the cells were exposed to a minimal-cytotoxic concentrations (IC10) of the chemicals during differentiation for 10 days, as determined by the AlamarBlue assay. After exposure, mRNA was extracted and the gene expression of the selected genes was analyzed by using RT-qPCR. All three neurotoxic chemicals downregulated the expression of most of the 30 genes. Concomitantly, the fraction of neurons in the cultures as well as the number of neurites per cell were significantly reduced. The fact that the downregulation of the genes correlates with structural changes further strengthens the significance of these genes for differentiation in this model. VPA displayed the greatest effect on the fractions of the neural populations as well as the number of neurites per cell followed by MeHg and ACR. VPA was also the substance that affected the selected mRNA markers the most in terms of log2(fold change) values, further supporting the indication that there is a correlation between the expression of markers and the structural readouts.

In conclusion, our results indicate that studying the expression of selected genes in the C17.2 cell model could be a potential tool to screen for DNT chemicals. The selected genes can be screened with optimized primers using RT-qPCR, a method that most laboratories have access to. The C17.2 cell line can be differentiated in a 2D-system without additional plate coating and with the addition of only two growth factors in the medium, making it an easy, fast and cheap model. The use of such models could help speed up the initial screening of substances, possibly alert for chemicals that need to be further studied in more sophisticated models.

**Paper III – Acrylamide alters CREB and retinoic acid signaling pathways during differentiation of the human neuroblastoma SH-SY5Y cell line**

The aim of this paper was to use the SH-SY5Y cell line to evaluate if ACR exposure affected differentially expressed genes and enriched signaling networks during neuronal differentiation. First, we performed a whole genome RNA-sequencing of the SH-SY5Y cells during differentiation for 3, 6, and 9 days and compared to the gene expression of undifferentiated cells. Downstream analysis of the differentially expressed genes was performed using IPA software (IPA, Ingenuity Systems, Redwood City, CA). As mentioned earlier, according to the literature, the SH-SY5Y cells can be differentiated by using various agents generating different neuronal phenotypes. Interestingly, we found that differentiation with 1 μM of RA induced differential expression of some cholinergic markers, e.g. choline O-acetyltransferase (CHAT), the vesicular acetylcholine transporter SLC18A3 and acetylcholinesterase (ACHE). It has been shown that some dopaminergic markers (tyrosine hydroxylase, dopamine receptor D2 and 3 (DRD2 and DRD3), and dopamine transporter (DAT)) can be upregulated during differentiation
with RA followed by phorbol esters. Interestingly, three dopaminergic markers were significantly upregulated using only RA, i.e. neurogenin 2 (NEUROG2), monoamine oxidase A (MAOA) and DRD2. Taking this to account we might have a mixture of both cholinergic and dopaminergic neurons in our differentiated cultures. However, one would have to verify that but looking at functional endpoints for the different phenotypes.

The IPA analysis showed that two enriched neuronal pathways were the RAR activation network and the CREB signaling in neurons pathway. We selected the differentially expressed genes in these pathways after 9 days of differentiation and looked at their expression after ACR exposure. We chose two different concentrations of ACR based on multiple assays monitoring cytotoxicity, cell death, cell number and neurite outgrowth. We had previously seen that low concentrations of ACR, starting at pM concentrations, increased proliferation and attenuated differentiation in comparison with equivalent control cells, after 6 days of differentiation as described in paper I. The same pattern was seen after 9 days of exposure to nM concentrations of ACR during differentiation (we did not look at lower concentrations in these assays). The two non-cytotoxic concentrations of ACR that were chosen for further analysis by RT-qPCR, i.e. 1 μM and 70 μM, both increased resorufin fluorescence without generating any cell death (as measured by PI staining). We calculated the amount of cells, the number of neurites per cell and the total neurite length for these two concentrations. Both concentrations significantly increased the number of cells while significantly decreasing the number of neurites per cell and mean neurite length. The cell and neurite counting also confirmed that the increase in resorufin fluorescence and ATP content was not caused by increased metabolic respiration due to e.g. cellular stress, but an actual increase in the number of cells in the cultures. Hence, both concentrations indicated that they were indeed non-cytotoxic but significantly reduced neuronal functional endpoints.

Both concentrations of ACR significantly altered the expression of several of the genes involved in the two pathways. For the CREB signaling pathway there were 16 genes that were significantly changed after exposure to 1 μM or 70 μM of ACR (18 including CREB1 and BDNF, which was not included in the original pathway generated by IPA). Nine of the genes were the same for the two concentrations. For the RAR activation pathway, 13 genes were significantly differentially expressed after exposure to both 1 μM and 70 μM of ACR. The expression of the same genes was affected by both concentrations. A few commonly expressed neuronal markers were added to the panel. The neuronal markers were additionally chosen due to their importance for neuronal differentiation in the SH-SY5Y cells (i.e. markers for cholinergic (CHAT) and dopaminergic (DRD2, MAOA)) neurons. We also selected markers that had been shown to form protein adducts with ACR in previously published literature (i.e. synaptotagmin 1 (SYT1)) or genes that had been shown to be affected by ACR exposure (i.e. MAOA and FGF1). We also added genes closely correlated with the two major pathways selected from IPA, i.e. BDNF, TrkB and CREB1. There were 6 genes that were significantly changed after exposure to 1 μM of ACR and 7 genes after exposure to 70 μM of ACR. CREB1 was not differentially expressed compared to undifferentiated cells but was investigated because of its central part of the CREB signaling pathway. ACR significantly increased the expression after exposure to both concentrations. The fact that ACR changed the expression of both neuronal and phenotype specific genes further strengthens the idea that ACR should be classified as a compound inducing DNT.

Methods such as RT-qPCR can be valuable when trying to evaluate and identify environmental risk factors from chemicals by studying the change in expression of specific genes that are
associated with neurodevelopmental disorders, e.g. autism or ADHD. It can also be an important tool when trying to evaluate the mechanism of action for a specific chemical. Our results indicate that the effect of ACR on neuronal differentiation in the SH-SY5Y cell line that we showed in paper I, is at least partly connected to changes in both the RAR activation and the CREB signaling in neurons pathways. Any chemical that alters the expression of CREB or BDNF signaling could have severe consequences since alterations to these pathways have been linked to disorders such as autism both in vitro and in vivo. The fact that ACR showed alterations to both pathways at non-cytotoxic concentrations indicates that there might be more knowledge to gain from these pathways. These pathways might be able to give more insight to the mechanism of ACR DNT. More research is needed to be able to safely put a tolerable daily intake limit on ACR intake during pregnancy. Especially since the adverse effects of ACR has been shown to be dose-dependent and accumulate over time.

Paper IV – Altered mRNA Expression and Cell Membrane Potential in the Differentiated C17.2 Cell Model as Indicators of Acute Neurotoxicity

The hypothesis for this paper arose from previous studies from our research group generated for the ACuteTox project, which was a framework program aimed to optimize an alternative toxicity test strategy for estimation of acute systemic toxicity. In this paper we wanted to investigate if cultures of differentiated C17.2 cells, consisting of both neurons and glia cells, could alert for chemicals that were known to induce acute toxicity by a neural MOA. The aim was also to evaluate if the C17.2 cell model could be used instead of the primary aggregating rat brain cell culture, that has previously been shown to be the best model for the evaluation of acute toxicity with a neural MOA in the ACuteTox project. We investigated this by using six compounds from the ACuteTox project. Of the six chemicals, three were neurotoxins, i.e. atropine sulphate monohydrate, nicotine, strychnine, and three chemicals that was considered as non-neurotoxic, i.e. acetylsalicylic acid, digoxin and ethanol. Ethanol is regarded as neurotoxic but human LC50 correlated well with the IC50 results from the 3T3-NRU assay performed in the project and hence, we did not expect our model to be able to identify it as a neurotoxic compound. The cells were differentiated for six days prior to exposure to non-cytotoxic concentrations of the chemicals. We assessed the CMP with the FMP assay during acute exposure. We also investigated the mRNA expression of selected markers for neural phenotypes and cellular stress, i.e. βIII-tubulin (an early neuronal marker), GFAP (astrocyte marker) and heat shock protein 32 (HSP32, also known as heme oxygenase-1 (HO-1), a marker for cell stress/cell survival). The results from both assays were used as neurofunctional endpoints to establish if the cells responded to the toxicants in a concentration-dependent manner at these non-cytotoxic concentrations.

Both assays could distinguish between the neurotoxic and non-neurotoxic compounds in the C17.2 cell model. The CMP assay detected that all three neurotoxic compounds induced a depolarization in the same range as the estimated lethal blood concentration for these compounds and at lower concentrations than the ones that decreased the number of viable cells by 50% in the neural red uptake assay in the mouse fibroblast cell line 3T3/NRU. No changes in the CMP were observed when the cells were exposed to the three non-neurotoxic compounds. For the analysis of the protein markers, 1/10 of the IC50 concentration that had been determined from the 3T3/NRU assay was used, and exposure proceeded for 48 hours. For all of the three neurotoxic compounds, at least one of the markers was significantly affected in its expression.
However, the concentrations did not alter morphology of the cells when inspected under the light microscope after exposure to the same concentration after 48 hours. Consistently, there was no effect on the mRNA expression after exposure to the non-neurotoxic chemicals.

In conclusion, the C17.2 model system was able to identify all three of the neurotoxic compounds selected in this study, while not giving any alert for the three non-neurotoxic compounds. The neurotoxic compounds affected both neurofunctional endpoints studied, i.e. CMP and mRNA expression. Taken together this suggests that the combination of studying CMP and selected mRNA markers could be useful for identification of compounds that induce acute toxicity by neural MOA. The simplicity, robustness and relatively low cost of the test system, and at the same time avoiding the ethical issues that comes with animal sacrifice, make it attractive as a tool for high-throughput toxicity screening.
Conclusions and outlook

During the last decade or so there has been an increasing effort being put into setting up systems for evaluation of DNT. A number of different model systems have been put into a testing battery including the use of stem cell/progenitor cell based methods. As mentioned before, using a battery of tests is necessary to cover the extreme complexity of neurodevelopment. Despite all of the efforts made in this field, the science surrounding test battery development is in an early state. There are good, robust methods for studying some aspects of neurodevelopment (e.g. neural proliferation, apoptosis, migration and neurite morphology) but there are also other parts of neurodevelopment that are less covered (e.g. glial differentiation, neuronal network formation, electrical network activity and sex specific neurotoxicity related to hormones). Understanding the physiology behind these complicated functions is a requirement that might enable establishment of AOPs for these important aspects in the future. In this thesis, some of the more well-known and established endpoints were used to evaluate if the two cell lines SH-SY5Y and C17.2 could be used to predict acute and developmental neurotoxicity. Emphasis was put on studying DNT-related effects of ACR by using these cell models and evaluating if these cell models could be used to gain insight on possible mechanisms of ACR induced DNT. The conclusions of this work are the following:

- Both cell lines included in this thesis can be used to study important neurofunctional endpoints with regards to acute and developmental neurotoxicity. The cell lines can be differentiated in a 2D-system without additional plate coating and with the addition of only two growth factors for the C17.2 cells or RA for the SH-SY5Y cells. This makes these cell models easy, fast and cheap to use. The use of such models could help to speed up the initial screening of substances, possibly indicating alerts that needs to be further studied in more sophisticated models.

- ACR affected both proliferation and differentiation in two different neuronal cell models. Both neuronal and the glial phenotypes in the C17.2 cell line were affected by ACR exposure. ACR attenuated differentiation at fM concentrations, i.e. concentrations that are 7 orders of magnitude lower than the estimated plasma concentration in the fetus. The fact that ACR seemed to attenuate differentiation at such low concentrations is alarming and should be further evaluated. The daily tolerated intake of ACR during pregnancy might need to be revised.

- The differences between the two cell lines should be further evaluated in regards to their sensitivities to ACR. The difference might be able to provide some insight to the toxicological mechanism of ACR.

- By using a limited number of mRNA markers that are important for neural differentiation, the C17.2 cell line could possibly be used to screen for alerting DNT chemicals.
Differentiation of the SH-SY5Y cells in serum free medium together with N2-supplements and 1 μM of RA showed a significantly increased expression of genes associated with both cholinergic and dopaminergic neuronal phenotypes.

Two of the enriched neuronal pathways during RA induced differentiation of the SH-SY5Y cells according to IPA were the RAR activation and the CREB signaling in neurons pathways. Both of these pathways were affected by non-cytotoxic concentrations of ACR after 9 days of differentiation/exposure. By looking further into these networks, we might be able to gain some mechanistic insight to the MOA of ACR DNT.

Using the C17.2 cell line, the CMP assay was proven to be a promising neurofunctional endpoint when screening for acute neurotoxicity. The CMP assay displayed higher sensitivity than the 3T3-NRU cytotoxicity assay and showed an improved correlation to human lethal blood concentrations.
Populärvetenskaplig sammanfattning


ska kunna forma ett korrekt kommunikationsnätverk mellan sig. Skador på dessa kan leda till alltifrån kognitiva nedsättningar till mer subtila förändringar hos människor och djur. Vi studerade också hur kemikalierna påverkade uttrycket av gener som är viktiga för utvecklingen och mognaden av hjärnans celler. Under de senaste åren har det utvecklats effektivare metoder för studier av arvsmassan (genomet), vilket gjort det mer tillgängligt för forskare att studera uttrycket av ett större antal gener samtidigt, eller till och med hela genomet. Vi har i våra studier använt oss av helgenomsstudier under mognadsutvecklingen av cellerna och har identifierat gener som är viktiga för hjärnans utveckling. Uttrycket av dessa gener har vi sedan studerat efter att cellerna exponerats för kemikalier. Fördelen med att identifiera färre, men kritiska, gener är att det blir billigare och mer anpassat för studier som kräver hög genomströmning av ett stort antal kemikalier. Vi studerade även hur kemikaliexponeringen påverkade proteinuttrycket av neurala proteiner såsom det neuronspecifika proteinet βIII-tubulin och det mer gliacellsspecifika proteinet GFAP. På så sätt kunde vi också se ifall kemikalierna förändrade förhållandet mellan de olika celltyperna.

Acknowledgment

Anna! Thank you for accepting me as a PhD student. You inspire, motivate and push me, exactly like a good supervisor should do. You have not only helped me in my development towards becoming a better researcher, but you have also always had my best interest at heart and given me great advice when it comes to life in general. I will always cherish that. I would also like to give a special thank you to my co-supervisor Anda. I’m so grateful that I met you at that Swetox conference in 2015. Since then we have published one (soon two) articles together and I have had the luxury of adding you as a co-supervisor for my PhD education. I have not only gained good advice and knowledge from you when it comes to my research, but I have also gained a good friend that I know I’ll keep for life.

Secondly, I would like to thank my former colleague and group member, Jessica. Since the day I started at the department you have been so supportive, kind, helpful and a true friend. I am so happy that I have gotten to know you both on a personal and a professional level. You are indeed an incredible human being and one of the sweetest persons I know. Thank you for everything.

Thirdly, I would like to thank all of the other PhD students, teachers and staff at the department (present and former). You have all made the department a welcoming place to work at. A special warm thank you to Anna-Lena and Marie-Louise, without your help and guidance these years would have been so much harder.

Till min familj, jag älskar er! Tack för att ni alltid har stöttat mig och funnits där när jag har behövt råd, en kram eller bara en knuff i rätt riktning. Mamma, pappa och Viktor, ni är mina bästa vänner. Utan er, inget jag! Farmor och farfar, jag är så otroligt tacksam för att ni alltid ställt upp under alla år med kärlek, goda råd, mat och boende. Kan inte göra annat än att hålla med farmor om att ”det är mysigt att tycka om”. Leo, tack för den kärlek och det lugn du gett mig under den här perioden. Och tack för korrekturläsning. För alltid min Aqua Aura!

To all of my beautiful friends! I have been fortunate enough to meet so many beautiful and wonderful people during my life. Some of you are more like family than anything else. Thank you for putting a smile on my face and for having my back through thick and thin. I love you guys. Life would be so incredible dull without you!
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