Proteomic Characterization of Induced Developmental Neurotoxicity

HENRIK ALM
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Abstract

The developing brain goes through a number of developmental periods during which it displays an increased sensitivity to exogenous disturbances. On such period is the so called “Brain growth spurt” (BGS) which in humans takes place starting from the third trimester of pregnancy and throughout the first few years of life. The corresponding period in rats and mice is the first postnatal weeks. Exposure to relatively modest concentrations of the brominated flame retardant PBDE-99 during the second week of life in mice causes a more or less permanent impairment in the ability of the animals to adjust properly to environmental changes at adulthood. This “late response on early exposure” reflects the long-term consequences of disrupting the developing brain during a sensitive time period.

The cellular mechanisms underlying the behavioral effects are far from clear. To address the initial damage occurring around the time of exposure, the approach used in this thesis is to use proteomics to analyze the effects of PBDE-99 on protein expression soon (24 hours) after exposure of the neonatal mouse on postnatal day (PND) 10. The thesis comprises the effects on the proteome in three distinct brain parts: cerebral cortex, striatum and the hippocampus. In addition, an in vitro model was developed and used to evaluate the PBDE-99 effects on cultured cerebral cortex cells from embryonic rat brains.

Gel-based proteomics (2D-DIGE) coupled to MALDI- or ESI-MS has been used throughout for the proteomics experiments, but other techniques aimed at analyzing both proteins and mRNA have also been used to better characterize the effects.

Even if the protein complements expressed by the different brain parts and separated with 2D-DIGE are seemingly similar, the effects are apparently specific for the different brain regions. In hippocampus, PBDE induces effects on proteins involved in metabolism and energy production, while the effects in striatum point towards effects on neuroplasticity.

PBDE-99 changes the expression of cytoskeletal proteins in the cerebral cortex 24 hours after exposure. Interestingly, in vitro exposure of cerebral cortex cells to a PBDE-99 concentration in the same order of magnitude as in the in vivo neonatal brain also induces cytoskeletal effects, in the absence of cytotoxicity. This may suggest effects on regulatory aspects of cytoskeletal dynamics such as those involved in neurite sprouting.

This thesis also addresses the problems involved in presenting proteomics data. Many of the available methods and approaches for presenting transcriptomics data are not suitable for isoform rich protein data. Modifications of existing methods and the development of a new approach (DEPPS) is also presented. Most importantly, the thesis presents the application and usefulness of proteomics as hypothesis generating techniques in neurotoxicology.

Keywords: Proteomics, Brain Development, Neurotoxicity, 2D-DIGE, Brain Growth Spurt, Polybrominated Diphenyl Ether, Neonatal, Striatum, Hippocampus, Cerebral Cortex, Parkinsonism, Dyskinesia

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Till Ellen
THE BLIND MEN AND THE ELEPHANT

John Godfrey Saxe

It was six men of Indostan
To learning much inclined,
Who went to see the Elephant
(Though all of them were blind),
That each by observation
Might satisfy his mind.

The First approach'd the Elephant,
And happening to fall
Against his broad and sturdy side,
At once began to bawl:
"God bless me! but the Elephant
Is very like a wall!"

The Second, feeling of the tusk,
Cried, "Ho! what have we here
So very round and smooth and sharp?
To me 'tis mighty clear
This wonder of an Elephant
Is very like a spear!"

The Third approach'd the animal,
And happening to take
The squirming trunk within his hands,
Thus boldly up and spake:
"I see," quoth he, "the Elephant
Is very like a snake!"

The Fourth reached out his eager hand,
And felt about the knee.
"What most this wondrous beast is like
Is mighty plain," quoth he,
"'Tis clear enough the Elephant
Is very like a tree!"

The Fifth, who chanced to touch the ear, Said: "E'en the blindest man
Can tell what this resembles most;
Deny the fact who can,
This marvel of an Elephant
Is very like a fan!"

The Sixth no sooner had begun
About the beast to grope,
Then, seizing on the swinging tail
That fell within his scope,
"I see," quoth he, "the Elephant
Is very like a rope!"

And so these men of Indostan
Disputed loud and long,
Each in his own opinion
Exceeding stiff and strong,
Though each was partly in the right,
And all were in the wrong!

MORAL.

So oft in theologic wars,
The disputants, I ween,
Rail on in utter ignorance
Of what each other mean,
And prate about an Elephant
Not one of them has seen!
“See first, think later, then test. But always see first. Otherwise you will only see what you were expecting. Most scientists forget that.”

Douglas Adams (1952 - 2001)
List of papers in the thesis

I. **Proteomic evaluation of neonatal exposure to 2,2',4,4',5-pentabromodiphenyl ether**
   *Environ. Health Perspect.* 2006;114, 254-259

II. **Exposure to brominated flame retardant PBDE-99 affects expression levels of cytoskeletal proteins in neonatal mouse cortex**
    Alm H, Kultima K, Scholz B, Nilsson A, Andrén PE, Fex-Svenningsen Å, Dencker L, Stigson M.

III. **Proteomic analysis of striatum in parkinsonian and dyskinetic non-human primates**
     *PLOS ONE* 2008 Feb 13;3(2):e1589

IV. **In vitro neurotoxicity of PBDE-99; immediate early and concentration dependent effects on protein expression**
    *Submitted*

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Neurofunctional deficits and potentiated apoptosis by neonatal NMDA antagonist administration
Fredriksson A, Archer T, Alm H, Gordh T, Eriksson P.
Behav Brain Res. 2004 Aug 31;153(2):367-76

Normalization and expression changes in predefined sets of proteins using 2D gel electrophoresis: a proteomic study of L-DOPA induced dyskinesia in an animal model of Parkinson's disease using DIGE
BMC Bioinformatics. 2006 Oct 26;7:475

Molecular targets and early response biomarkers for the prediction of developmental toxicity in vitro
Stigson M, Kultima K, Jergil M, Scholz B, Alm H, Gustafson AL, Dencker L.
Altern. Lab. Anim. 2007 35, 335-342

Global neuropeptide analysis in embryonic quail diencephalon
Submitted
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<td>2-DE</td>
<td>Two-dimensional gel electrophoresis</td>
</tr>
<tr>
<td>2D-DIGE</td>
<td>Two-dimensional difference gel electrophoresis</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>BGS</td>
<td>Brain growth spurt</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CGN</td>
<td>Cerebellar granule neuron</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>Cy₂</td>
<td>Cyanine</td>
</tr>
<tr>
<td>Cy₃</td>
<td>Indocarbocyanine</td>
</tr>
<tr>
<td>Cy₅</td>
<td>Indodicarbocyanine</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichloro-diphenyl-trichloroethane</td>
</tr>
<tr>
<td>DEPPS</td>
<td>Differential expression in predefined protein sets</td>
</tr>
<tr>
<td>DHB</td>
<td>Dihydrobenzoic acid</td>
</tr>
<tr>
<td>DIGE</td>
<td>Difference gel electrophoresis</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DRM</td>
<td>Detergent-resistant membrane</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothretil</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma amino butyric acid</td>
</tr>
<tr>
<td>GD</td>
<td>Gestation day</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>GSEA</td>
<td>Gene enrichment analysis</td>
</tr>
<tr>
<td>HUPO</td>
<td>Human proteome organisation</td>
</tr>
<tr>
<td>ICAT</td>
<td>Isotope-coded affinity tags</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>Isobaric tags for relative and absolute quantification</td>
</tr>
<tr>
<td>Ip</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto encyclopedia of genes and genomes</td>
</tr>
<tr>
<td>LTQ</td>
<td>Linear trap quadrupole</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MEEBO</td>
<td>Mouse exonic evidence based oligonucleotide</td>
</tr>
<tr>
<td>MeHg</td>
<td>Methyl mercury</td>
</tr>
<tr>
<td>MeSH</td>
<td>Medical subject headings</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------------------</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>MudPIT</td>
<td>Multidimensional protein identification technology</td>
</tr>
<tr>
<td>Mw</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NL</td>
<td>Non-linear</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NMRI</td>
<td>National Maritime Research Institute</td>
</tr>
<tr>
<td>NTD</td>
<td>Neural tube defects</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBDE</td>
<td>Polybrominated diphenyl ether</td>
</tr>
<tr>
<td>PBDE-99</td>
<td>2,2',4,4',5-pentabromodiphenyl ether</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated biphenyl</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFOA</td>
<td>Perfluoroctanoic acid</td>
</tr>
<tr>
<td>PFOS</td>
<td>Perfluoroctanoic sulphate</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMF</td>
<td>Peptide mass fingerprinting</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>PTM</td>
<td>Posttranslational modification</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>REACH</td>
<td>Registration, evaluation and authorization of chemicals</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcribed</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulfate</td>
</tr>
<tr>
<td>SILAC</td>
<td>Stable isotope labeling by amino acids in cell culture</td>
</tr>
<tr>
<td>STEM</td>
<td>Short-time series expression miner</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
</tr>
</tbody>
</table>
Introduction

The genomic revolution, which culminated in the sequencing of the human genome [1], raised great hopes that the identification of disease-relevant genes would pave the way for new pharmaceutical formulations and treatment strategies. Early estimates predicted over 100 thousand genes in the human genome [2], while other more moderate calculations predicted 60-70 thousand genes [3]. After it was realized that the human genome only consists of some 22 thousand protein coding genes, which is comparable to the mouse genome and only slightly more than the roundworm C.elegans which we intuitively attribute lesser complexity, it was apparent that the number of genes does not explain the apparent differences between mouse and man (or worm for that matter).

The vast majority of genes encode information for the production of proteins, essential polymers involved in almost all biological functions. Being the building blocks of cells and the true messengers of biology, the study of protein involvement in life and disease has over the years attracted much attention in the scientific community. These studies have also led to important findings regarding disease mechanisms and treatments.

The traditional approach of studying one gene or protein has been successful in many areas of research. These analyses are often based on prior hypotheses regarding mechanisms of action or biological function. However, focusing on just one analyte at a time will give a very limited insight into the dynamic situation in the cell, and a more comprehensive view of the biological situation is missing. It is like in Saxe’s old tale about the blind men and the elephant [4], cited above. Biological systems are simply too complex and we will end up knowing a lot about trees and being clueless about the forest.

Since its inception in the mid 1990’s, the use of proteomics techniques has grown rapidly. Defined as “any large-scale protein based systematic analysis of the entire proteome or a defined sub-proteome from a cell, tissue or entire organism” [5] this new approach to biology was enabled by the emerging availability of complete genome sequences and instruments for protein and peptide analysis. In contrast to the traditional approach focusing on detailed studies of one or a few proteins, proteomics takes a more comprehensive and systematic approach to understand biological systems. As a consequence, proteomics is discovery-based rather than hypothesis driven and is therefore not constrained by prior knowledge in the field of enquiry.
While the genome of an organism is relatively stable and finite over the entire lifetime, the proteome is dynamic and constantly changing in response to developmental events and to external stimuli. This increase in complexity from genome to proteome is both due to alternative splicing of the gene transcripts [6] and to the notion that 80% of all proteins are subject to a range of more than 300 different types of post translational modifications (PTMs) [7]. As a consequence, proteome analysis is much more complicated than genome analysis, especially in higher eukaryotes where the proteome complexities far outweighs the technologies currently available for a complete proteome characterization [5]. However, even if proteomics as such is a large-scale platform, much of the attraction lies in the possibilities to use the proteomics tools on selected populations of proteins in specific circumstances [8], thus contributing directly to mechanistic or functional questions in biology and medicine.

Large-scale studies of proteins in applied neurosciences are not new, but despite the potential impact of these studies they are not yet fully established as high value contributors in the scientific community. In the early days, the large scale genomic and proteomic techniques were criticized for producing a lot of “dirty data”, and for drawing biological conclusions based on poorly annotated gene or protein data. Much of the criticism was valid. However, even if some of the techniques have matured and today deliver high quality data they are not fully established as high value contributors in research. As is shown in this thesis, used properly the large-scale techniques complement other techniques in molecular biology and add another quality to the answers to questions in biology and medicine.

This thesis describes large-scale protein studies applied to developmental neurotoxicology. The recurring theme in the papers is the use of protein methods ideally describing proteome wide expression changes. However, and importantly, the methods are applied to biological questions which have clear (behavioral) phenotypes but where we also lack fundamental knowledge about initiation and progression of the phenotypic traits. The developing brain and the negative consequences of early developmental disturbances for later functionality are today greatly acknowledged [9, 10]. However, the effects on the developing brain of low-dose exposure to environmental contaminants and other chemicals to which humans and animals are constantly exposed are in many cases appreciated, but not well understood. Bridging behavioral data to meaningful mechanistic hypotheses is a formidable task, likely requiring joint efforts using different methodologies and design approaches. Here we have used a proteomics approach, where we identify early changes (24 hours) in protein expression as a result of a single oral dose of the brominated flame retardant PBDE-99 during a sensitive period of brain development in mice.

The introductory part of this thesis will give a theoretical background to the biological and technical issues in the thesis. The materials and methods
section describes the tools and techniques used in the experiments. It will also review the technical achievements in paper III. The Results and discussion section summarizes the biological results from the papers and gives future directions for further studies in the field.

Toxicology and development

An important fallacy in the toxicologist’s mantra “The Dose Makes the Poison” is the failure to take sensitive sub-populations into account. This fallacy is perhaps most obvious if you consider differences in sensitivity between different developmental ages. The idea that there are developmental periods during which the embryo is particularly sensitive to exogenous disturbances underlies the whole subject of embryotoxicology. The event that provided an immense stimulus to the development of the subject was the recognition that thalidomide is a potent human teratogen [11]. Thalidomide, which was marketed in the 1950’s as a sedative and as a drug for morning sickness in pregnancy, was removed from the market in 1961 after it was associated with an epidemic of severe birth defects. 15000 babies worldwide were born with missing limbs [12]. Pictures of children deformed by thalidomide aroused the public, and new laws and regulations were passed in many countries which led to stringent drug safety requirements. However, even after the thalidomide incident the full dimensions of developmental toxicity remained largely unappreciated. For example, despite centuries of anecdotal observations a distinctive label for prenatal ethanol toxicity, Fetal Alcohol Syndrome, did not appear until the mid 1970s [13]. The metals mercury and lead for which there are anecdotes about fetal toxicity since ancient times may also exemplify how little recognition, until very recently, was accredited the special sensitivities of children [14].

When prescribing a drug during pregnancy and the period of nursing today we are often aware of the risks involved for mother and embryo/fetus/child thanks to national and international monitoring programs which aim at detecting adverse drug responses [15]. However, for environmental chemicals, where we often are unaware of the extent of exposure via food, drinking water, house dust or other routes, the risks are more uncertain.

Environmental chemicals

The rapid industrial growth that followed the Second World War resulted in an enormous influx of new chemical substances, together with frequently unchecked releases of pollutants. For most (if not all) of them, fundamental knowledge about intrinsic properties and effects on human and environ-
mental health was missing. With time, some measures were taken to limit the “use and abuse” of chemicals. However, the risks for humans and wildlife associated with environmental exposure to chemicals came to public interest only with the publication of Rachel Carson’s “Silent Spring” in 1962 [16]. In the book, Carson presented a dire scenario about the use of pesticides and other toxicants and their effects on the environment. The uproar that followed the publication of the book eventually lead to a strengthening of the regulation of pesticides, and in many respects lead the way towards an awareness of chemicals’ impact on the environment.

Despite this “awareness” we have witnessed a number of disasters, where humans and animals have been exposed to significant amounts of chemicals as a result of industrial release e.g. the Minamata disease [17] and the Bhopal disaster [18]. However, while true disasters they are, from a toxicological perspective, not very cumbersome since usually there is a defined population which is exposed for high levels for a limited time. The effects are often dose-related, and when the causative agent is found, further exposure is avoided. The long-term effects may of course be dramatic and extend to involve future generations, but the causative agent and the exposure episode are well defined.

In contrast, continuous low-dose exposure to environmental chemicals via food, drinking water, air or other routes, represent what may be defined as “silent exposures” i.e. exposures where the effects are not immediate, but become apparent years or decades after the exposure [19]. It can be compared to the process of carcinogenesis, where the chemical exposure and cellular damage occurs years or decades before the emergence of the clinical manifestations [20]. Considerable attention is being focused on the neurodevelopmental effects of pre- and postnatal exposures to a large number of environmental contaminants. This interest is intensified by the growing recognition of an increase in the incidence of neurodevelopmental disabilities, such as learning disabilities and Attention deficit hyperactivity disorder (ADHD) [21]. A growing body of experimental research and epidemiological data indicate that environmental toxicants may play a role in this increase [22, 23]. It has also been hypothesized that exposure of the developing brain to toxic environmental agents during windows of vulnerability during early life may be an important contributor to the causation of neurodegenerative diseases such as Parkinson’s disease and Alzheimer’s disease [24].

1 Unfortunately, this information is still missing for many chemicals. This is the reason for the European Community regulation REACH (1907/2006), which gives greater responsibility to industry to provide safety information on the substances and manage the risks for chemicals.
Brain development begins early in human gestation through the process called neurulation. Functionally, this process can be divided into a primary phase during which the neural plate changes rapidly in shape and rolls into a tube, forming the brain and rostral spinal chord and a second phase during which the caudal region of the spinal chord is formed [25]. This progresses both rostrally and caudally in a zipper-like fashion. The neural tube formation is complete at approximately gestation day (GD) 26-28 in humans [26]. From here cell division, migration, differentiation, maturation and synapse formation occur in well ordered sequences with variable timing in different brain parts. Neural migration and myelination continue through infancy and well into adolescence [27], and thus it is arguable that brain development takes place over a period of 20 years or more. In between these time points a myriad of developmental events are passed, all of them potentially vulnerable to disturbances. However, that is not to say that all developmental events are equally sensitive.

A great body of scientific evidence has shown that there are multiple periods of increased vulnerability to toxic insults in the developing system spanning from early gestation to adolescence in humans and experimental animal models (reviewed in [28]). These “critical periods” or sensitive periods during brain development are highly plastic, brief defined periods of development during which the brain is particularly vulnerable for disruption by environmental influences [29]. Adverse insults during these periods result in diverse outcomes ranging from morphological changes that emerge during early embryogenesis that may be incompatible with life, to ultrastructural or molecular changes which appear during any developmental time point and are associated with functional deficits.

Failure of proper neural tube closure can result in a relatively common class of human malformations known as neural tube defects, in which the neural tube remains open locally (spina bifida), or in the cranial region (encephaly, anencephaly) or in worst case scenario throughout the entire neuraxis (chraniorachischisis totalis) [9, 25]. It has been shown that exogenous disturbance during this period using for instance the antiepileptic drug valproic acid increases the risk for neural tube defects (NTDs) tenfold [30], and is perhaps the first manifestation of developmental neurotoxicity. However, other structurally and chemically diverse teratogens, such as the antiepileptic drug carbamazepin, the metal arsenic and the antibiotic drug trimethoprim also results in an increased incidence of neural tube defects [31-33], suggesting that this outcome is due to multiple factors.

It is arguable that just as different mechanisms may lead to similar spectra of structural malformations, different mechanisms may lead to similarities in behavioral manifestations (Table 1). This may also relate to the fact that although there are a multitude of possible outcomes of exposure to a devel-
opmental toxicant, the functional expression of the damage is limited to a finite set of behavior paradigms. Accordingly, it may be difficult to find behavioral tasks that reflect the underlying neural processes that have been disrupted by the exposure.

The same is of course true for structural aberrations, where the relative ease to detect the aberrations underlying certain behavior varies with the resolution of the techniques used. Considering that many environmental developmental toxicants (such as PCBs, lead, mercury) influence behavior in the absence of malformation induction at higher doses [34], other techniques which assess changes at the molecular level may be better suited for finding mechanistic underpinnings for a behavioral disturbance.

### Table 1. Developmental neurotoxicants which induces adverse behavior effects in adult rodents after exposure during the BGS. PND denotes postnatal day.

<table>
<thead>
<tr>
<th>Neurotoxicant</th>
<th>Class</th>
<th>Exposure regimen</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>Bioallethrin</td>
<td>Pesticide</td>
<td>PND10-16</td>
<td>[35, 36]</td>
</tr>
<tr>
<td>DDT</td>
<td>Pesticide</td>
<td>PND10</td>
<td>[37, 38]</td>
</tr>
<tr>
<td>Deltamethrin</td>
<td>Pesticide</td>
<td>PND10-16</td>
<td>[35]</td>
</tr>
<tr>
<td>Diisopropylfluorophosphate</td>
<td>Organophosphate</td>
<td>PND3, 10</td>
<td>[39]</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Recreational drug</td>
<td>PND10</td>
<td>[40, 41]</td>
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<td>Iron</td>
<td>Metal</td>
<td>PND3-5, 10-12</td>
<td>[42-44]</td>
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<td>Ketamine, MK-801</td>
<td>NMDA-R antagonists</td>
<td>PND10</td>
<td>[40, 41]</td>
</tr>
<tr>
<td>MeHg</td>
<td>Organometallic compound</td>
<td>PND10</td>
<td>[45, 46]</td>
</tr>
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<td>PCBs</td>
<td>Polychlorinated biphenyls</td>
<td>PND10</td>
<td>[47, 48]</td>
</tr>
<tr>
<td>PFOA</td>
<td>Organofluorine</td>
<td>PND10</td>
<td>[49, 50]</td>
</tr>
<tr>
<td>PFOS</td>
<td>Organofluorine</td>
<td>PND10</td>
<td>[49]</td>
</tr>
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</table>

### Brain growth spurt

One critical period of brain development is the stage when nerve cells send out axons and dendrites, establish neurotransmission systems etc, the so-called “brain growth spurt” (BGS) [51]. This spurt starts during the third trimester in humans and spans through the first few years of life. The corresponding period in rodents is the first few weeks postnatally [51, 52] (Figure 1). This reflects the differences in brain development between rodents and humans. Rodents are considered altricial; born at relatively unde-
veloped stages and with many neurodevelopmental events taking place post-natally. Humans, however, would be classified as altricial based on the immaturity of body and motoric skills, while classified as precocial due to the advanced development of perceptual systems at birth [53].

The BGS is a period during which the brain grows at an accelerated rate because newly differentiated neurons throughout the brain are rapidly expanding their dendritic arbours to provide the required surface area to accommodate new synaptic connections. This is also a period of rapid myelin formation during which most afferent pathways are already present in their target areas, although their distribution and synaptic targets are still immature [54]. Amidst this multitude of differentiation and growth, there is also cell death. Unsuccessful neurons that do not receive the proper input or trophic signals are removed by apoptosis [55]. This physiologic cell death is a natural pruning process by which the developing brain deletes cells that are not needed for further development and function.

Using primarily rodents, it has been shown that developmental exposure to a number of environmental pollutants, pharmaceuticals and recreational drugs (e.g. ethanol) during this brain development period give rise to adverse behavioral effects in adult animals (Table 1). The effect on behavior from several, but not all, of these agents has been related to an altered cholinergic system function and/or receptor density [35, 38, 39, 47-49, 56]. For ethanol [57] and ketamine [58] the changes in behavior have been correlated with a potentiation of the physiologic cell death to include cells that should not have been deleted. Collectively, the studies show that the BGS is a sensitive period of brain development which is susceptible to disturbances from a wide range of chemicals.

Animal models of human neurotoxicity

Modelling a human disease is very difficult, and very often the models extend to merely display some of the disease symptoms. This is especially true for animal models for diseases of the brain, such as Parkinson’s disease and Alzheimer’s disease. One underlying reason is of course that we lack a comprehensive understanding of disease initiation and of the mechanisms underlying disease progression. Another reason is the model itself.

For a number of apparent reasons (ethical, legal etc.) humans cannot be used for toxicity testing or for disease modeling. There is also considerable ethical constraint to using non-human primates, even if they are sometimes used (paper III in this thesis is an example). Although there are exceptions, rats and mice are by far the most used species in neurotoxicity testing, certainly developmental neurotoxicity [59]. There are a number of reasons for this, including vast knowledge of macro-and micro-neuroanatomy, neurophysiol-
ogy and that assessments of behavior are well-mapped [53, 59]. Yet another reason is related to the development of the brain.

**Figure 1.** A. Comparative velocity curves of the Brain growth spurt for selected species. Rat and Mouse are postnatal brain developers. Guinea pig is a prenatal brain developer with the major part of the velocity curve outside (left) of the graph. **B,** Cortical progenitor cells follow a distinct pattern of development both in vivo and in vitro. The generation of the three cell types occurs in a distinct pattern. **C,** Brain-region specific neurogenesis patterns in rat. **D,** Isolation of cells for primary cell cultures (paper II and IV). GD is gestational day, PND is postnatal day and P0 is day of partus/birth. Based on information provided in [9, 52, 60].
Comparative aspects of brain development

Research efforts in developmental and evolutionary biology have shown that the timing and sequence of early events in brain development are remarkably conserved across mammals [53]. However, there are vast differences in elaboration and relative sizes across mammalian species (e.g. cortical regions, limbic areas) as well as differences in the relationship of birth to the maturational state of the brain [61].

One way of looking at brain growth relative to birth is dividing species arbitrarily into prenatal, perinatal and postnatal brain developers. Based on brain wet weight (which is a crude parameter), humans are here classified as perinatal brain developers [51]. The mouse and the rat, which are mostly used in this thesis, are both postnatal brain developers [51, 52] but have gestation times that differ by three days (mice 19.5d, rats 22.5d). Are the three additional days mainly employed for brain development, meaning that cross-species comparison between rat and mouse is as simple as subtracting the extra days of gestation for the rat? Beside the more crude method to compare brain development used by Dobbing [51, 52], a recent approach has been presented by Finlay and Darlington where data from ten mammalian species (including human) is used for 102 neurodevelopmental events [53, 61, 62]. Interestingly, comparing mouse and rat brain development using this model it seems as if the developmental events in general differ 1-3 days between the species (except eye-opening which differs almost six days counted post conception) [62]. For instance, according to the model the peak of neurogenesis in the hippocampal subregions CA1 and CA2 in mouse is at GD15.2 while the same period in rat is GD17.51. Thus, arbitrarily adding 3 days for species comparison between rats and mice is not correct. Rather, depending on anatomical region of interest there are differences in timing of events, reflecting that the mammalian brain development is heterogeneous.

In contrast to rats and mice, the spiny mice and the guinea pigs are “pre-locial2” brain developers or pre-natal brain developers, meaning that they are relatively independent at birth (Figure 1). They are born with their eyes open, and even shed their baby teeth in-utero [53]. This considerable brain development before birth makes them quite unsuitable (certainly impractical) for modeling human brain development, especially aspects that are dependent on experience. However, their prolonged development in utero may make them useful models for “nature vs. nurture” studies.

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1 The Finlay & Darlington model uses the term post conception (pc) which is normally defined as days post conception (dpc) or embryonic day (Ed) in prenatal studies. Most of the studies on prenatal and perinatal PBDE exposures in mice and rats use the term gestational day (GD) which can roughly be transformed to 1pc+0.5.

2 By convention, evolutionary biology ranks animals into altricial or precocial species. Altricial species are born in a relatively immature state and has a prolonged postnatal dependency, while precocial species are born with very mature nervous systems and undergo little postnatal brain development.
In conclusion, finding a good model for human brain development is difficult. Due to economical and ethical constraints, other primates are rarely used as models. Although rat and mouse brain development is, not least from a temporal perspective, strikingly different from human brain development they are the most used models in developmental neuroscience. *In vivo* and *in vitro* mice and rat models are used throughout this thesis.

**Polybrominated diphenylethers**

Besides the compounds listed in Table 1, another class of substances that induces a disturbed adult behavior if the developing mouse or rat is exposed during the neonatal period is the group of Polybrominated flame retardants (PBDEs). The penta-brominated congener PBDE-99 is used as model substance for neonatal brain disruption in this thesis.

After the PCBs were banned from production in the late 1960’s, due to concern over their toxicity and persistence in the environment, the brominated flame retardants (BFRs) were established as the new major chemical flame retardants [63]. PBDEs took a prominent role of the market, and were marketed in three technical mixtures with varying degrees of bromination: penta-, octa- and deca-BDE. They were used in large quantities in electronic equipment, furniture and plastics to prevent fire. With time it was evident that all PBDEs are potentially prone to bioaccumulate in the environment by leaching out from the flame retardant-treated products [64].

Human sources of PBDE exposure include the occupational setting, the diet and the indoor environment. Among foods, fish has the highest content of PBDEs, followed by meat and dairy products [65]. A breast-milk monitoring program in Sweden has shown a significant increase of PBDE levels in human breast milk from 1972 to 1997 [66], but a recent study observed decreasing levels of PBDE in samples from 1998 and later [67]. Mother’s milk in the United States currently contains the highest levels of PBDEs worldwide [68], and has been reported to be 10-100 times greater than in Sweden [69]. Infant exposure to PBDEs via breast milk is of great concern, especially since lactation in many mammals coincides with the rapid development of the brain, the BGS [51]. Due to the high levels in breast milk it has been estimated that a breastfed infant would be exposed to approximately 306ng/kg/day, as compared to 1 ng/kg/day for adults [70]. Although the body burden in humans is well documented, there is almost no information on possible adverse effects in humans from PBDE exposure, including developmental neurotoxicity [71]. Thus, any inference on potential risk for adverse neurodevelopmental effects of PBDEs in humans has to rely on animal data.
PBDE related behavioral studies

A considerable number of behavioral studies of developmental PBDE exposure have been performed (Table 2 and reviewed in [71]). Although it is beyond the scope of this thesis to discuss them in detail, some aspects will be brought to attention here.

Most of the behavioral studies of developmental PBDE exposure have been performed using a single oral dose of PBDEs around PND10 in mice, which coincides with the peak of BGS [51, 52]. This is also the model we have used for our in vivo studies on developmental PBDE-99 neurotoxicity throughout this thesis (Paper I and II).

The other major exposure-regime is pre-natal exposure, usually single-dose or intermittent exposure from around PND6. There is a certain discrepancy regarding model use, where studies for postnatal exposure consistently have used rat models, whereas peri- and postnatal exposures use both species but most often the mouse (Table 2).

Another point to be made is regarding the doses. The administered doses required for behavioral effects in the adult are generally lower when a prenatal (gestational) approach is used. In addition, for persistent (sometimes even worsening) behavior effect, the postnatal dosing-regime is, as it seems, required, while prenatal exposure gives transient effects on behavior. The underlying reasons for this are not clear, but may need further attention and an increased focus.

24 hours after PBDE-99 exposure on PND 3, 10, or 19, approximately 3-5‰ of the administered dose is found in the brain of the neonatal pups [72]. Mice exposed on day 3 or 10 show impaired spontaneous behavior as adults, while no effects are found after exposure on day 19 [72], suggesting that day 19 is outside of the window of sensitivity. To date, all PBDE congeners except the octabrominated BDE-183 and the decabrominated BDE-209 have shown persistent adverse effects in spontaneous locomotor behavior in the adult after exposure on PND10. BDE-183 and BDE-209 on the other hand, induce long term effects in spontaneous behavior after exposure on PND3. A possible explanation for the difference in sensitivity period for BDE-209 is caused by metabolism of the congener, and that the metabolites left in the organism around PND10 are the causal agents for neurotoxicity [73]. A similar explanation has been used for the effects of BDE-99 in PND3 mice, making the causal agent for neurotoxicity the remaining BDE-99 or its metabolites present around PND10 [72]. No similar explanation has been proposed for BDE-183.
Table 2. Mouse and rat behavior studies after developmental PBDE exposure

<table>
<thead>
<tr>
<th>PBDE</th>
<th>Exposure regimen</th>
<th>Species*</th>
<th>Comment</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>DE-71</td>
<td>Postnatal</td>
<td>Rn</td>
<td></td>
<td>[74]</td>
</tr>
<tr>
<td>BDE-47</td>
<td>Prenatal</td>
<td>Rn</td>
<td></td>
<td>[75]</td>
</tr>
<tr>
<td></td>
<td>Perinatal</td>
<td>Rn</td>
<td></td>
<td>[76]</td>
</tr>
<tr>
<td></td>
<td>Postnatal</td>
<td>Mm</td>
<td></td>
<td>[77, 78]</td>
</tr>
<tr>
<td>BDE-99</td>
<td>Prenatal</td>
<td>Rn</td>
<td></td>
<td>[79, 80]</td>
</tr>
<tr>
<td></td>
<td>Perinatal</td>
<td>Rn</td>
<td></td>
<td>[81]</td>
</tr>
<tr>
<td></td>
<td>Perinatal</td>
<td>Mm</td>
<td></td>
<td>[82, 83]</td>
</tr>
<tr>
<td></td>
<td>Postnatal</td>
<td>Mm</td>
<td></td>
<td>[77, 84-87]</td>
</tr>
<tr>
<td></td>
<td>Postnatal</td>
<td>Mm With MeHg or PCB-52</td>
<td></td>
<td>[45, 88]</td>
</tr>
<tr>
<td></td>
<td>Postnatal</td>
<td>Rn</td>
<td></td>
<td>[89]</td>
</tr>
<tr>
<td>BDE-153</td>
<td>Postnatal</td>
<td>Mm</td>
<td></td>
<td>[90]</td>
</tr>
<tr>
<td>BDE-183</td>
<td>Postnatal</td>
<td>Mm</td>
<td></td>
<td>[91]</td>
</tr>
<tr>
<td>BDE-203</td>
<td>Postnatal</td>
<td>Mm</td>
<td></td>
<td>[91]</td>
</tr>
<tr>
<td>BDE-206</td>
<td>Postnatal</td>
<td>Mm</td>
<td></td>
<td>[91]</td>
</tr>
<tr>
<td>BDE-209</td>
<td>Postnatal</td>
<td>Mm</td>
<td></td>
<td>[92-94]</td>
</tr>
<tr>
<td></td>
<td>Postnatal</td>
<td>Rn</td>
<td></td>
<td>[95]</td>
</tr>
<tr>
<td></td>
<td>Postnatal</td>
<td>Mm With PFOA</td>
<td></td>
<td>[50]</td>
</tr>
</tbody>
</table>

*Mm – Mus musculus, Rn – Rattus norvegicus

In addition to changes in spontaneous behavior, cognitive impairments manifested a decreased spatial memory in Morris swim maze [96] and impaired working memory in radial arm maze [45] has been seen after PBDE-99 exposure on PND10.

Large-scale protein studies of the mammalian brain

The brain is arguably the most complex tissue of the mammalian species. Considerable heterogeneity is observed in the nervous system on all levels examined. A huge number of histological regions, defined nuclei, sub-nuclei and even individual cell clusters can be identified. This structural (and functional) heterogeneity continues on the cellular level. The human brain is estimated to contain some $10^{12}$ neurons and perhaps ten times more glia cells [97]. Several thousand cell types can be identified based on shape, function and biochemical properties [98]. At any given moment, these different cell types express different parts of the genome, giving a considerable genomic complexity. The complexity of the proteome is increased even further by alternative splicing, PTMs and degradation products (sometimes referred to as the “degradome” [99]). The levels of different protein entities display a considerable heterogeneity throughout the body. The concentrations of dif-
ferent proteins in serum can vary by a factor of $10^{10}$ [100] while conservative estimates of the dynamic range in tissues are in the order of $10^6$ [101]. This protein complexity outweighs the technologies currently available for protein characterization. As a consequence, elucidating the protein complement (proteome) of the mammalian brain is a major challenge.

From proteins to proteomes

Studies of proteins have historically focused on the analysis of single molecules. Although this approach has served biology and medicine well, the shift to large-scale analysis was necessary to in the long run generate fundamental knowledge of biological systems. It is simply not likely that single protein analysis en masse will generate a comprehensive understanding of the biological complexity.

The development of protein sequencing by Frederick Sanger [102], which gained even more momentum with the phenylisothiocyanate sequencing chemistry development by Per Edman in 1949 [103], in many respects paved the way for modern protein analysis. However, peptide sequencing was performed manually, and the complete sequencing of a protein required huge amounts of peptides from several digests of the target protein. Also, an array of different proteases was needed to collect a redundant or overlapping set of peptide fragments to cover the whole protein sequence. The automation of the Edman sequencing in 1967 [104], and a commercial instrument greatly improved the situation, but it was still a relatively low-throughput technique requiring large amounts of sample and having difficulty in sequencing short (less than 50 amino acids) peptides [105]. At this time, a protein was almost always isolated and purified based on the basis of its biochemical activity, before the covalent structure (amino-acid sequence and modifications) was elucidated in detail. Once this was known, interaction partners was found and analysed in detail starting a new round of search for interacting molecules [106]. Although successful in many respects, disadvantages of this approach include the need for large amounts of sample, and tedious isolation procedures.

The focus shift from single proteins to the entire protein complement of a cell or tissue, defined as the proteome [107], dates back almost 40 years with the development of two-dimensional gel electrophoresis (2-DE). Early attempts combining naive isoelectric focusing with pore gradient sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [108] were promising, but the technique that is still in use today originates from the work by Patrick O’Farrell, who used two dimensional gel electrophoresis to separate proteins from Escherichia coli in 1975 [109]. Shortly thereafter Klose [110] and Scheele [111] used the technique to separate mouse and guinea pig proteins respectively. However, even if the technique allowed the proteins to be separated, they could not be identified. To overcome this prob-
lem, it was necessary to develop some kind of sensitive protein-sequencing technology [106, 112]. Until the 1990s, however, identification of proteins separated on 2D gels was limited to only the most abundant proteins [113]. In 1993 Henzel published the first paper where proteins separated with 2-DE were indentified with mass spectrometry (MS) [114, 115]. Together, these two events, the development of 2-DE and the development of MS, provide the foundation of all further milestones in proteomics.

Mass spectrometry

Even if mass spectrometry (MS) can be dated back to the early 1900s, it was not until the 1980s and on a larger scale 1990s that MS started to play an important role in the biosciences [113]. A major leap forward for proteomics in general, but certainly for the applicability of 2-DE, was the development of two ionization techniques in the late 1980’s; electroSpray ionization (ESI) [116] and matrix-assisted laser desorption ionization (MALDI) [117]. These “soft” ionization methods which generates ions from large non-volatile analytes, such as proteins and peptides, without significant analyte fragmentation [118] have contributed significantly to the development of the proteomics field. In fact, John Fenn and Koichi Tanaka were awarded the Nobel Prize in chemistry 2002 for their pioneering work with the ESI and MALDI technique respectively.

MALDI-MS

After enzymatic cleavage of the proteins using a selective protease (e.g. trypsin, which hydrolyzes only the peptide bonds in which the carbonyl group is contributed either by an Arg or Lys residue), MALDI generates protonated molecules in a gas-phase by co-crystallizing analyte molecules with a matrix prior to irradiation of the crystals with nanosecond laser pulses [119]. The matrix is usually a small organic molecule with absorbance at the wavelength of the applied laser. Matrices of α-cyano-4-hydroxycinnamic acid or dihydrobenzoic acid (DHB) are most commonly used for biological samples. Matrix differences include the amount of energy they impart to the peptides during desorption and ionization, which is related to the fragmentation they cause [113]. In paper I we used the α-cyano-4-hydroxycinnamic acid matrix, which generally leads to the highest sensitivity in MALDI.

ESI-MS

In papers II, III and IV we used the ESI ionization technique. In contrast to the MALDI technique, ionisation is in ESI performed with the analyte in a solution which is pumped in microliter-per-minute flow rate through a hypodermic needle, and forms an aerosol with a mist of small droplets. An uncharged carrier gas such as nitrogen is sometimes used to help nebulize the solvent thus producing the solvent droplets. As the solvent evaporates, the biomolecular ions are formed [113, 114].
Global protein analyses of the mammalian brain

To date there have been numerous efforts to generate proteome maps of the human and rodent brains, e.g. [120-124]. Beside minor biological insights, these studies have perhaps most importantly highlighted the need for standardization to ensure robustness and reproducibility between laboratories.

One such effort is a program called HUPO Brain proteome project (HUPO BPP) under the patronage of the Human Proteome Organisation (HUPO). This program aims at mapping the proteome of the human and mouse brains in healthy, neurodiseased and aged status with focus on Alzheimer’s and Parkinson’s disease [125]. Even if highly needed, the project is very ambitious and will probably take many decades to complete.

Proteomics studies of the developing brain

Few studies have been performed to analyze the proteome of the developing brain (e.g. [126, 127]). One of the first efforts was performed by Fountoulakis in 2002 [123] using aborted human fetuses. A more recent approach describes the effects on brain proteins of disrupting the neonatal rat brain [128]. Most importantly, this study shows the variable response of protein-isoforms over the course of development. In principle, the authors identified 4 groups of proteins after exposure on PND6 to Phenobarbital (GABA\(_{\alpha}\)R agonist or dizocilpine (NMDAR antagonist): early-, late-, transient-, and stable proteins. The early group was proteins found on PND7, but diminished or disappeared at later stages, while the late group proteins were not visible on PND7, but appeared at later ages (PND14, PND35 or PND56). Transient proteins only appeared at a specific age, while stable proteins were largely unchanged at all ages. Interestingly, comparing the expression with a 24 hour exposure in adult animals (PND55) the authors discerned proteins that were affected after neonatal exposure (PND6) but not after adult exposure (PND56). The fact that very few proteins that were altered acutely following drug exposure in infancy (PND6) were not differentially expressed in adulthood was by the authors seen as an indication of the underlying sensitivity of the developing brain [128]. Interesting analogies can be made with neonatal PBDE-99-exposure, where the (behavioral) sensitivity is centered around PND10, and exposure on PND19 is outside the apparent window of increased sensitivity [96].
Aims of the thesis

When the aims were formulated there was an extensive and expanding literature on the spatial and temporal expression of genes during brain development, but little was known about the protein expression, and even less about the potential changes in protein expression that might be the result of exposure to various chemicals. Therefore, the aims of this thesis were to explore the mechanisms with which environmental influences (i.e. chemicals) affect the normal development of the mammalian brain.

More specifically, the objectives of the thesis were:

- To explore the effects on the mouse brain proteome a short time (24 hours) after neonatal exposure to PBDE-99
- To develop and evaluate a relevant *in vitro* model for neurotoxicology studies
- To apply proteomics techniques on *in vitro* models to add to the knowledge on *in vivo-in vitro* comparisons.
- To generate strategies to present large-scale protein data
Methods

Several different methods are used in this thesis, all aiming at providing molecular information about events in cells and tissues under different physiological conditions. Even if I set out to study proteins, complementary non-protein methods have been added along the way. These include techniques for mRNA analysis such as Quantitative real-Time PCR (qRT-PCR) and cDNA microarrays. Even if the relative contribution of these techniques to the thesis is small, I have chosen to describe them in some detail below. Methodological aspects and results of paper I-IV are included in this section as well as a general discussion about data presentation in proteomics. The biological results of Paper I, II and IV are found in the results and discussion section.

2-DE or not 2-DE – Choice of proteomics method

A number of global protein detection methods are available, all of them with merits and weaknesses (Table 3). The methods are traditionally divided into gel-based (2-DE, 2D-DIGE) and gel-free (MuDPIT, ICAT, ITRAQ), where the gel-free methods in this list all are MS based. Another gel-free method; protein arrays has intentionally been left out from the table, since it is not global in the sense that it is dependent on the availability of antibodies. Studies where gel-free and gel-based methods have been compared with similar samples indicate that the methods are complementary. Although their analytical windows overlap, each of them has exclusive sets of proteins that were not identified by the other techniques [129-132]. Thus, from that perspective, an ideal proteomics experiment should include both gel-based and gel-free methods.
Table 3. Pros and cons using different global protein detection methods. Modified from [133].

<table>
<thead>
<tr>
<th>Technique</th>
<th>Merits</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2-DE</strong></td>
<td>Detects PTMs</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td>Differential expression</td>
<td>Low global separation</td>
</tr>
<tr>
<td></td>
<td>Visualizes isoforms</td>
<td>Solubilisation problems</td>
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<tr>
<td></td>
<td>Native separation possible ¹</td>
<td></td>
</tr>
<tr>
<td><strong>2D-DIGE</strong></td>
<td>Detects PTMs</td>
<td>Low global separation</td>
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<tr>
<td></td>
<td>Improved sensitivity</td>
<td>Solubilisation problems</td>
</tr>
<tr>
<td></td>
<td>Improved differential expression</td>
<td>Non-native proteins only</td>
</tr>
<tr>
<td></td>
<td>Visualizes isoforms</td>
<td></td>
</tr>
<tr>
<td><strong>MuDPIT</strong></td>
<td>Sensitive</td>
<td>No differential expression between samples</td>
</tr>
<tr>
<td></td>
<td>High resolution</td>
<td>PTMs not detected (easily)</td>
</tr>
<tr>
<td></td>
<td>No labeling necessary</td>
<td>Low throughput</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-native proteins only</td>
</tr>
<tr>
<td><strong>ICAT</strong></td>
<td>Sensitive</td>
<td>Proteins with no cysteines are missed</td>
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<tr>
<td></td>
<td>Quantitative</td>
<td>Differential elution of isotope pairs</td>
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<tr>
<td></td>
<td>Detects PTMs</td>
<td>Non-native proteins only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Complicated MS-spectra due to addition of biotin groups</td>
</tr>
<tr>
<td><strong>iTRAQ</strong></td>
<td>Sensitive</td>
<td>Complex spectra</td>
</tr>
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<td></td>
<td>Quantitative</td>
<td>Low throughput</td>
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<tr>
<td></td>
<td>Detects PTMs</td>
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<td>4-plex possible</td>
<td>Isoforms interfere with isobaric tags</td>
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<td><strong>SILAC</strong></td>
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<td>Enables combination with activity assays</td>
<td>Low throughput</td>
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<tr>
<td></td>
<td></td>
<td>Non-native proteins only</td>
</tr>
</tbody>
</table>

Animals and tissues
This thesis includes tissues and cells from three different mammalian species. In paper I, hippocampi and striata dissected from 10-days old NMRI mice were used. In paper II the cerebral cortices from 10-days old NMRI mice were used and primary cell cultures were prepared from cerebral cortices from GD17 Sprague Dawley rat embryos. Paper III is the result of col-

¹ Blue Native PAGE (BN-PAGE) is possible, where native proteins are separated.
laboration with a French and Chinese team using striata from the non-human primate *Macaca fascicularis*. In paper IV we used primary cell cultures which we prepared from cerebral cortices dissected from GD 21 Sprague Dawley rat embryos.

**Experimental design**

Paper I was designed according to the recommendations given by the DIGE manufacturer at that time. Accordingly, it does not include a dye-swap design as it was assumed that the dyes had similar labeling biochemistry. For each individual sample, frozen tissue from three pups was pooled before cell lysis generating six pools (three controls and three treated). We added extra unlabelled protein to our gels for identification purposes. To our knowledge, there were no publications at the time where the effects of adding extra unlabelled protein material to a DIGE gel had been analyzed. To account for potential unwanted effects of this addition, we added one extra gel to each experiment (hippocampus and striatum). Randomized dye-swap designs for individual samples were used in paper II and III, while a dye-swap loop design for individual samples was used in paper IV.

**Sample preparation**

The adequacy of the data in experiments involving biological samples is crucially dependent on the sample preparation. If this fact is overlooked, it is of no importance how sensitive or reliable your assay is, the data will still be of poor quality. Ideally, unprocessed samples are used without any time delay and without confounding effects of storage, temperature fluxes, handling and potential contamination. However, since this is not practically possible, care must be taken to minimize the effects of these parameters on the data.

Working with proteins or RNA, the main focus of the sample preparation is to avoid enzymatic degradation by proteases or nucleases which will affect the reproducibility of the experiments. These enzymes are always present to some extent, but are released in huge amounts during tissue or cell damage such as when grinding tissues or disrupting cell membranes to release proteins or RNA. In addition, post-mortem degradation of proteins and peptides may cause breakdown of target proteins and peptides and thus cause sample heterogeneity seen as poor reproducibility. A number of methods have over the years been utilized to minimize sample degradation. The most basic ones are quick dissection of tissues of interest and then putting them in liquid nitrogen, dry ice (frozen carbon dioxide) or even ordinary ice. Other methods used, particularly in MS-based experimental setups, are using microwave irradiation for quick inactivation of degradation enzymes [134, 135]
and using knock-out mice lacking certain forms of peptidases [136]. However, even if all samples should always be treated optimally, the effects of improper sample preparation will depend on the resolution of the assay or experiment. Degradation studies of proteins and peptides have shown that proteins are more resilient to degradation than peptides, and that post-translational modifications (PTMs) are affected already after 1 minute post-mortem in mice [137].

In papers I and II, the brain tissues (hippocampus, striatum and cerebral cortex) were quickly dissected and put in eppendorf tubes covered in dry ice within 2 minutes, ensuring minimal protein degradation. Before analysis, the frozen tissues were individually put in lysis buffer (see protein solubilisation) containing protease inhibitors (Complete Mini, Roche Diagnostics). Thereafter the samples where kept on ice whenever possible to minimize protease activity.

In paper III, dissection of the different brain regions were performed on ice with the brain immersed in ice-cold saline (0.9%) in less than 15 minutes. This is a quite extended time, and effects from protein degradation are possible [137]. The striatum (combining caudate nucleus, putamen and nucleus accumbens, across the rostrocaudal extent of the structure) was dissected from each hemisphere, immediately frozen at -45°C in isopentane and then stored at -80 °C.

In paper IV the cultured cells were lysed on the culture plates to minimize handling and potential degradation.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2-DE) is based on the separation of proteins in two dimensions based on differences in iso-electric point, pI, and size (mw). The first dimension separation, the iso-electric focusing (IEF) was in O’Farrells original paper from 1975 maintained dynamically with ampholytes [109]. Later, Bengt Bjellqvist immobilized the pH gradient within a gel matrix using buffering monomers called immobilines [138]. The second dimension is traditional SDS-PAGE, where the proteins are separated according to their molecular mass (Mₚ). Proteins and SDS form complexes composed of micelles connected by short polypeptide segments [139]. Since the SDS to protein ratio in these complexes are 1,4g SDS/g protein and the protein charges are masked by the negative charge of SDS, the complexes have a relatively constant net negative charge per mass unit. This negative charge will make the complexes migrate towards the anode in an electric field.
Sample preparation for 2-DE

The success of any 2-DE experiment is dependent on a number of factors, and the ignorance of any of them will give gel-images with suboptimal resolution, resulting in poor reproducibility between gels and between experiments. The importance of proper sample preparation has already been mentioned. For 2-DE, the sample preparation is focused on three fundamental issues: cell disruption, removal of interfering substances (such as ions or lipids) and solubilisation of the proteins [140]. As a consequence of cell disruption and liberation of proteins and other cell constituents, proteases and other substances interfering in the electrophoresis process are released in the sample. Proteases are usually removed using protease inhibitors, such as Pefabloc® serine protease inhibitor, or EDTA [141]. Ideally, protease inhibitors against all possible proteases should be used to give the samples optimal protection against protease degradation. We have consistently used Complete Mini (Roche Diagnostics) which protects against serine, cysteine and metalloproteases. Removal of interfering compounds can be performed using a standard PCA precipitation, but is nowadays most often performed using sample preparation kits, which precipitates the proteins, and after a series of washes dissolves them in a new buffer optimised for 2-DE. Salt-concentrations higher than 100mM in the sample will give streaking and other problems related to poor electrophoresis [142]. This is particularly important if in-gel rehydration 2-DE is performed (see IEF). Using the cup-loading technique this problem is smaller, but salt will still give you longer electrophoresis times due to increased conductivity of the IEF gel. In all our studies we have used the 2D Clean-up kit (GE Healthcare) to remove interfering substances.

Prefractionation

One of the major obstacles when working with proteins is that, so far, there is no “protein PCR-method” with which you can amplify your protein of interest. You simply have to work with what is biologically available. Thus if you are looking for e.g. signaling proteins which may be less abundant by several orders of magnitude compared to structural proteins, you have to find a way to reduce the sample complexity. This can be done by sample prefractionation. In Paper II we wanted to increase the resolution by prefractionating the proteins, enabling us to analyze cytosolic (soluble) and membrane (less soluble) proteins separately. This sequential extraction procedure was accomplished using the 2D-fractionation kit (GE Healthcare).
Solubilisation of proteins

It would of course be preferential to analyze the proteins under native conditions, since it would give the opportunity to conserve the unique three-dimensional structure of each protein and also enable studies of protein interactions. However, although preferential, it would also create a number of practical problems. Keeping the native conditions would mean that the proteins existed in several conformations, which would result in very complex 2D patterns. In addition, native protein aggregates would be too big to enter the gels, resulting in poor reproducibility.

To overcome these problems, denaturing conditions are used for 2-DE. The choice of lysis (or sample solubilisation) buffer strongly influences the resulting sample composition and the types of proteins you end up separating in your electrophoresis. Any suitable 2-DE sample buffer, however, must contain chaotropes, detergents and reducing agents [141]. In the numerous preparative studies before the experiments in paper I, we performed comparative studies using sample buffers containing variable concentrations of urea (and thiourea). It is generally believed that for optimum solubilisation of membrane proteins, a sample buffer consisting of 7M urea and 2M thiourea should be used [140]. However, we found that for our purposes a sample buffer only containing 9,6M urea worked as well (results not published), which also spared us from using the known carcinogen thiourea. In addition, the use of thiourea usually generates a vertical streaking in the acidic area, which gives poor resolution and blurred spots in the pH range below this streaking. As already stated, the sample buffer must consist of a detergent to prevent hydrophobic interactions between membrane domains, thus preventing protein loss due to precipitation or protein aggregation. The zwitter ionic detergent 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS) has proven to be one of the most all-round detergents for 2-DE.

The most commonly used reducing agent in 2-DE is probably dithiothreitol (DTT). It is added to the sample buffer to reduce the proteins and prevent re-oxidation of the proteins once reduced. Also, they cleave sulphide bonds thus achieving complete protein unfolding. Thus, in the first paper we used a sample buffer consisting of 9,6M urea, 4% CHAPS, 70mM dithiothreitol (DTT); 5% immobilized gradient (IPG) buffer, pH 3-10. However, in the following papers we separated the proteins in a much wider pH interval (3-10 or 3-11NL compared to 4-7 in the first paper) and in an interval including high pH. Since DTT is a weak acid (pK around 9) it means that it will become ionized at high pH and move towards the anode during the IEF leaving the proteins unprotected from oxidation. In general, this can be seen as horizontal streaking in the gels. To prevent this, we used DeStreak solution (GE Healthcare) instead of ordinary reductant in papers II, III and IV.
First dimension: Isoelectric Focusing (IEF)

The first dimension IEF has developed considerably since the pioneering studies of O’Farrell, but the general idea is the same; to separate the proteins based on differences in iso-electric point (pI). Originally performed using thin polyacrylamide gel rods in glass tubes with carrier ampholytes to create the pH gradient in the electric field [109], 2-DE is now performed using individual IPG drystrips with immobilised pH gradients. A number of different pH ranges is available from wide range strips (e.g. pH 3-10) to more narrow-range drystrips (e.g. pH 4.5-5.5). The choice of drystrip is of course dependent on the pIs of the proteins of interest. Narrow range drystrips give higher first dimension resolution and enables separation of spots in crowded areas. In addition, narrow range strips also allows higher protein loads, which enables identification of low-copy number proteins. From this it follows that the ideal 2-DE experiment would be performed using multiple sets of narrow-range IPG strips with redundant pH ranges covering the whole pH scale. However, such an experimental design would require huge amounts of starting material, be very tedious and difficult to analyze, and, not least, be very expensive. As a consequence, unless you are looking for a specific subset of proteins in a certain pH range, it is most common to use as wide pH range as possible. This enables separation and identification of proteins with a wide difference in pI.

The choice of pH range affects the separation characteristics. As discussed in the previous section, high pH ionizes the reducing agent DTT which makes it exceedingly difficult to get appropriate separation of high pI proteins. Very acidic proteins are also more difficult to separate.

Second Dimension: SDS-PAGE

The second dimension in 2-DE is a traditional SDS-polyacrylamide electrophoresis (SDS-PAGE). This technique separates the proteins based on differences in size ($M_r$). SDS and proteins form complexes, where proteins are covered in SDS in micelles in a ratio of 1.4g SDS per gram protein [139]. Since SDS masks the charge of the proteins, and the formed anionic complexes have a relatively constant net negative charge per unit mass [139], all proteins will migrate towards the anode. This migration through the polyacrylamide gel results in a size separation, where the smaller proteins travel faster, and will be found further down on the gel. By using pre-stained molecular weight standards, it is possible to estimate the $M_r$ of a protein by comparing the relative distance travelled by a protein with that of the standard proteins. However, for a more exact estimate, MS is needed.

It is easily conceived that the bigger the gel, the better the resolution. The same is also true for the first dimension separation; the longer the drystrip, the better the resolution. Depending on the commercial system used, there
are different drystrip and gel sizes. The work in this thesis is based on experiments using GE Healthcare (formerly Amersham Biosciences) equipment. They provide strips and gel formats of 7, 13, 18 and 24 cm. For optimal resolution, we have used 24 cm strips and gels throughout the experiments.

Protein detection

A wide variety of protein detection methods are used in the literature, which all of them have advantages and disadvantages. Ideally, your method of choice should have a low detection limit and a high signal to noise ratio. In addition, it should have a wide dynamic range, a wide linear relationship between protein quantity and staining intensity, be non-toxic, environment-friendly, easy to handle, have a long shelf-life and be cheap [143]. Evidently, no such method exists. For the papers in this thesis, we have used three methods, which will be briefly described below.

Coomassie Brilliant Blue

Staining with Coomassie Brilliant Blue is likely the most frequently used staining method for 2-DE. Since its introduction in 1963 [144], several improvements have been made to the staining protocols. Perhaps the most important improvement, certainly for reproducibility and sensitivity, was the introduction of Colloidal Coomassie Blue staining. In our studies we have consistently used a variant of the protocol by Neuhoff [145]. Advantages using this method include the high reproducibility and a wide linear relationship between protein and staining intensity. An important drawback is the relatively low sensitivity, at least compared to silver staining and the newer fluorescence stains (e.g. Deep purple™ and Sypro® Ruby).
Figure 2. Flowchart of a typical 2D-DIGE experiment. A. Biological samples are labeled with fluorescent dyes (Cy-dyes) prior to separation in two dimensions. Scanning at wave-lengths corresponding to the excitation maxima of the different fluorophores generates unique images for each sample. Images are matched and analyzed to identify spots (proteins) of interest before spot picking and MS analysis. B. Examples from papers III and IV showing the usefulness of spatial normalization. The 15% highest and lowest log₂Cy5/Cy3 are colored red and blue respectively. The top-left image (raw data) displays higher Cy5 values while the top-right shows higher Cy3 values. After spatial normalization (top-right image) the spot pattern is randomized. The same line of reasoning is applicable to the bottom panes.
Figure 3. Gel picture from paper IV, where a representative part of a DIGE-stained gel is compared with the same gel after ProQ-Diamond staining. The Pro-Q diamond stain detects phosphate groups attached to tyrosine, threonine or serine residues.

**Difference gel electrophoresis (DIGE)**

What has been described as a “quantum leap for 2D gel electrophoresis methodology” [143] was the development of the DIGE technique by Ünlü in 1997 [146]. DIGE is a technique used for labeling protein samples prior to electrophoresis, using size and charge matched fluorescent dyes (CyDye™ fluorors) with distinct spectral characteristics. Since a recent development of the technique [147], the protein samples are labelled with one of three different CyDyes (Cy2, Cy3, or Cy5). The labelled proteins are then mixed and separated on the same 2D gel [148]. The protein samples labelled with different CyDyes can subsequently be visualized separately by exciting the different dyes at their respective excitation wavelengths (*Figure 2*). This is generally accomplished by scanning the gels with a fluorescence scanner, which generates separate images depending on CyDye.
Stains for post-translational modifications

One of the major advantages of using 2-DE compared to other proteomics methods is the fact that post-translational modifications (PTMs) are readily visualized. However, even if you can visualize spot families and with MS identification confirm that they are variants of the same protein, it is impossible to deduce the isoform-specific modifications of the proteins with traditional stains. Since a few years back PTM-specific post-stains are available for a few modifications (e.g. phosphorylations, glycosylations) that give the possibility to visualize gel-wide differences in PTMs [149, 150]. In paper IV we used Pro-Q® Diamond (Molecular Probes) to visualize the fractions of the separated proteins that were phosphorylated (Figure 3).

Image analysis

Scanning

The scanning is very important and likely influences the result of 2D-DIGE experiments. Even so, very few studies (e.g. [151]) have focused on the scanning procedure and the importance of optimizing the laser power for each individual gel due to variations in loading amounts, dye and other technical variations. As a consequence, most groups (us included) use the traditional scanning procedure which is suggested by the dye manufacturer (GE Healthcare). In that procedure, one of the gels in the experiment is pre-scanned at a low resolution (500 μm), and the PMT (laser power) is adjusted to the level where no spots are saturated. That PMT level is then used to scan all the gels also at high resolution (100 μm). We have used this scanning procedure for all the gel-experiments in this thesis. Subsequent follow-up studies in our lab (unpublished data) have shown that this procedure is not optimal. Rather, each gel is unique and requires individual laser settings to give un-biased fluorescence from all three individual channels.

2D analysis softwares

To analyze the spot patterns on a 2D gel is a formidable task, and is nowadays always performed using computer softwares. Since the introduction of the first computer-based analysis systems (without a graphical interface) in the late 1970’s such as ELSIE [152, 153], TYCHO [154] and GELLAB [155-157], a number of commercial softwares designed for 2-DE image analysis have been developed. In 2007, seven commercial software products for 2D gel image analysis were available [158]. Even if I have some experience in using three of them (Delta 2D (DECODON), Dymension (Syngene) and DeCyder (GE Healthcare), all the image analyses in this thesis were performed using the DeCyder software.
Analysis

In general, the gel analysis has been performed in accordance with the instructions from the manufacturer of DeCyder (GE Healthcare). For all analysis softwares, the intentions are to minimize user biases i.e. minimize the effects on the analysis result that are dependent on changes and modifications that are introduced by individual users. However, no software is perfect. As a consequence, the spot detection and spot matching have in papers I-IV been manually verified for all the spots on all spotmaps. Although laborious, it significantly improves the adequacy of the data, and is highly recommended.

2D-DIGE data is in many ways similar to microarray data, which is why many recent approaches in 2D-DIGE data handling have been adopted from concepts originating in gene expression studies. The need for proper normalization of proteomics data has been shown previously by us [159] (Figure 2B) and others [160, 161]. Also, regarding statistical testing, different approaches are used to find differently expressed spots in 2D-DIGE experiments. Some use no statistical test, but identify their proteins of interest based on average fold-changes [162], while others use unpaired t-tests [163] or ANOVA tests [164]. In this thesis, aspects of t- and F-statistics have been used throughout. See individual papers for reference.

Data presentation in proteomics

One of the principal challenges to proteomics studies beyond the technical aspects of the experiments is how to present the data in an accessible way. Proteomics can generate enormous volumes of data that if presented tabulated or in lists may provide nothing but confusion to the reader. However, and importantly, this work starts already when planning the experiment as the way the data can be presented depends on the design of the experiment.

Figure 4 is an overview of the strategies commonly used to present proteomics expression data. From the overview it is evident that the experimental parameters strongly influence the complexity (in the biological sense) and the level of biological interpretation possible from the studies. In that perspective, the papers in this thesis show a development from single pair wise comparisons to more multifaceted comparisons where it is easier to confer effects on a systemic level.

Another major divider in proteomic analysis is how to deal with isoforms. One way is to consider all the proteins with the same MS-identity as one protein (as in e.g. [165, 166]). This “isoform-free” or genomic-like approach enables the use of traditional genomics techniques for large-scale analysis, such as gene ontology (GO) analysis, but does not appreciate isoform-specific effects. In addition, merging all spots originating from the same gene product but with different PTMs may well cause a misinterpretation of
the effect on the protein level [159]. This is especially true if not all protein isoforms from a particular protein is identified.

The other approach, which has been used consistently throughout the papers in this thesis, is where isoforms are considered unique identities. Although biologically superior, this creates a number of practical problems. All of the problems however, stem from the fact that there (to my knowledge) are no good GO [167] or GO-like tools (such as GSEA [168]) that takes into account redundant isoforms. This was solved in paper III with what is called “Differential Expression in Predefined Protein Sets” (DEPPS) [159].

DEPPS

Differential Expression in Predefined Protein Sets (DEPPS) is an adaption of the gene set tests previously suggested by Subramanian [168] and Tian [169]. In DEPPS, proteins are classified into groups (sets) based on biological function, involvement in biological processes and cellular localization. All proteins were manually categorized using the information provided, when available, by the Gene Ontology project [167] and related information from the scientific literature. A total of 137 sets were used; 70 predefined sets from the Molecular Signature Database (MSigDB, http://www.broad.mit.edu/gsea/) [168] and 67 manually annotated sets.

After normalization and ranking [159] the protein spots are plotted in a volcano plot. A volcano plot displays the measure of statistical significance of the change, lodsratio (for log-odds ratio) versus fold change. A high lodsratio indicates a higher chance of true differential expression compared to a low lodsratio. This method has been widely used for data from gene expression arrays [170] and we have used it throughout in our 2-DE studies. The lodsratio is used to rank all the spots in each set of interest and a p-value is assessed for each protein set. As a more in-depth analysis of the statistical issues underlying DEPPS is beyond the scope of this thesis, please see [159] for reference.
Figure 4. Overview of strategies commonly used to present proteomics data.
Initially the DEPPS analysis was based solely on lodsratio-derived ranking [159]. This approach is sensitive to proteins with high lodsratios, resulting in DEPPS sets containing such proteins to rank higher. In paper III the DEPPS analysis was modified from the original and used both lodsratio-based and consecutive-based ranking (Figure 6).

STEM

Short-Time Series Expression Miner (STEM) was originally designed for microarray derived data [171] and compares sequential groups, such as dose-response or time-dependent data. In papers III and IV we used it to generate profiles for protein data. Multiple testing in STEM model profiling was done using Bonferroni correction with a significance level of 0.05. Significant STEM profiles are calculated by comparing the number of proteins assigned to a profile to the number of proteins expected by a permutation test.

In papers III and IV we wanted to correlate the expression profiles to gene ontology analysis [172], but to incorporate isoforms in the analysis, we had to modify the GO-annotation. Assuming small functional differences between the isoforms of a given protein for a given GO-annotation, GO sets were created by adding indexes to the original IDs. Isoforms for e.g. Id2 were labeled Id2_a, Id2_b….Id2_n which enabled GO analysis.

Figure 5. Representative STEM images from paper III. One hour of L-Dopa treatment dramatically changes the expression of a large number of proteins in Parkinsonian Macaca fascicularis monkeys. One hour is not enough for cells to synthesize most proteins de novo, suggesting that the effects are for the most part due to changes in PTMs.
Figure 6. Overview of DEPPS analysis as performed in paper III. Rather than focusing on the proteins above an arbitrarily defined cut-off or fold-change, DEPPS takes all identified isoforms into account (red spots in the volcanoplot). Related proteins are classified into functionally associated sets which are compared in a pairwise fashion. To reduce possible sets effects from strongly differentially expressed proteins (high lod-ratios), two types of DEPPS analyses were conducted; constitutive ranking (R-score) and lodsratio-based ranking (L-score). The DEPPS table (bottom) shows RL-scores for striatal TCA-metabolism and aminoacid-associated protein sets in six pair-wise comparisons from the study of L-Dopa induced dyskinesia in parkinsonian macaques (paper III). # designates the number of proteins in the sets.
PCA

In paper IV the data was analyzed using principal component analysis (PCA) with the software SIMCA-P+ [173]. PCA is frequently used in exploratory analysis and is a good starting point for analysis of multivariate data. It reduces the dimensionality of the data by finding the orthogonal directions in multivariate space that represents the largest sources of variation, the so-called principal components (PCs). Thus, by expressing as much as possible of the variation in the data with just a few PCs, an overview of the data is provided showing how the observations are related and if there are any deviating groups or observations in the data (paper IV, Figure 1 and 3).

In SIMCA-P+, PCA is visualized using two different plots (Figure 7. Score plots represent the projections of the objects (e.g. treatment groups), while the loading plot shows the relations between the variables (e.g. normalized protein expression data). Objects that are projected close to each other in the score plot have similar characteristics, and those characteristics are defined in the loading plot. Thus, objects projected in the far right corner of the score plot have similar characteristics, and those characteristics are defined by the variables in the far right corner of the loading plot. Also, the further an object is from the axis origin, the more it contributes to the model generated by the PCA.

Figure 7. Score plot and loading plot from the larger dataset (n=816) in paper IV. The score plot shows the relation between the objects, while the loading plot displays the relations between the variables. The colored geometrical shapes in the loading plot display the distribution of the identified Dpyl protein family members (Dpyl1, Dpyl2, Dpyl3 and Dpyl5). Dpyl2 and Dpyl3 proteins show isoform specific effects.
Western Blot

Western blotting (or immunoblotting) is a technique used to verify the expression of a protein or the relative amount of the protein in different samples. Proteins are separated according to molecular weight using denaturing SDS gel electrophoresis and transferred to a membrane. The target protein is probed using a specific antibody and detected usually using chemiluminescence. In paper I we used the ECL Plex Western Blotting Detection System (GE Healthcare), which uses CyDye labeled secondary antibodies, thus enabling detection of two different antibodies simultaneously. The fluorescence signal is detected with a fluorescence scanner. In papers II and IV, ECL Advance Western Blotting Detection Kit was used. The principle here is that enzyme (e.g. Horseradish peroxidase) labeled secondary antibodies bind to primary antibodies that have targeted the protein of interest. The enzyme activates a substrate which then emits light. The signal is recorded on a photographic film.

Gene expression studies

Microarrays

Gene expression microarrays are small solid supports which contain hundreds to thousands of nucleotide probes immobilized at fixed locations to a surface. A specific RNA quantitatively hybridizes to each probe, which enables the expression measurement of thousands of genes simultaneously. There are two common microarray approaches; one-channel microarrays and two-channel microarrays. In two-channel microarrays, long sequences (cDNAs) or oligos (50-75 base pairs) are printed onto a glass slide. Two samples of RNA which have been labelled with different fluorescent dyes (e.g. Cy3 and Cy5) are hybridized to the slide. Since the fluorescent dyes have different excitation and emission wavelengths, the fluorescence signal from the samples can be independently determined with a laser scanner. The pixels from the resulting image are quantified and analyzed.

In paper IV we used two-channel microarrays of the MEEBO (Mouse Exonic Evidence Based Oligonucleotide) set containing a collection of 70mer 5’ amino modified oligonucleotide probes. In this set, a total of 35,302 probes targeting mouse genes are printed on two slides. The sequence divergence between species (rat RNA on mouse arrays) gives an increased risk of sequence mismatches during hybridization. Earlier cross-species microarray studies have adjusted for sequence divergence by excluding oligos where the sequences are not identical [174]. Here the mouse clones were blasted using cross-species megaBLAST (NCBI) against the rat genome,
using default parameters. Only clones with at least 95% sequence identity were used in the study.

Real-time PCR

The polymerase chain reaction (PCR) has greatly simplified the detection of nucleic acid molecules in biological samples. In theory, a single copy of a particular sequence can be specifically amplified and detected. Ideally, there is a quantitative relationship between amount of starting target sequence and amount of PCR product. However, increasing numbers of cycles is correlated to a higher variability in the product yield due to variations in for instance starting material and efficiency. The development of real-time quantitative PCR (qRT-PCR) by Higuchi et al. in 1992 [175] has to a large extent eliminated the variability traditionally associated with quantitative PCR since it measures amplification throughout the whole PCR reaction. In real-time PCR the amount of product formed, and the number of amplification cycles required to obtain a particular amount of DNA molecules is analyzed. This is performed by monitoring the fluorescence of dyes or probes introduced into the reaction which is proportional to the amount of product (double stranded DNA) formed. A number of fluorescent dyes and probes are available, but throughout this thesis the PCR experiments have been performed using SYBR green. This asymmetric cyanine dye is considered to be an excellent sequence non-specific reporter in real-time PCR, but may give rise to primer-dimer products [176]. We have controlled for these effects by melting curve analysis. Since primer-dimer products typically are shorter than the PCR products, their melting temperatures are lower, making them easily recognized. The real-time PCR approach used in this thesis (paper IV) is semi-quantitative, since it measures the relative mRNA expression for different samples.
Results and discussion

Since there were no good experimentally based hypotheses regarding molecular mechanisms of neonatal PBDE neurotoxicity, the whole idea underlying this thesis was to use hypothesis generating techniques (proteomics) to look for “functional biomarkers” of PBDE-99 exposure. The rationale was to take a snap-shot of the molecular processes taking place in the neonatal mouse brain 24 hours after PBDE-99 exposure. Papers I and II describe the effects on the proteome in discrete brain parts (hippocampus, striatum and cortex) of the neonatal mouse, 24 hours after exposure to PBDE-99. In paper IV, an *in vitro* approach was developed to assess the PBDE-99 induced effects on cultured cerebral cortex cells.

This result section describes papers I, II and IV (Table 4). Aspects of paper III are found in the materials and methods section.

Table 4. Overview of proteomics experiments

<table>
<thead>
<tr>
<th>Paper</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
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<tbody>
<tr>
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<td>Rattus norvegicus</td>
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<td><em>In vitro</em></td>
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<tr>
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<td>18</td>
<td>111</td>
<td>476</td>
<td>292</td>
</tr>
</tbody>
</table>

<sup>1</sup> The age of the monkeys used for the proteomics experiment was 3-5 years; average age was 4.4 years.

<sup>2</sup> Identified proteins after matching and spot quality control. More proteins were successfully identified in all experiments but were removed due to matching or spot quality problems.
Mechanisms of PBDE-99 induced developmental neurotoxicity

Considering the relative wealth of studies on PBDE induced behavior effects, there is only little information available on the molecular mechanisms. Even if you consider the published papers on all PBDE congeners (Figure 8) the mechanistic studies are still relatively sparse. It is of course arguable that behavioral studies per se can be “mechanistic” at least to the point that they can be specific towards specific brain parts, or even circuits.

![Mechanistic studies of PBDE-induced neurodevelopmental disturbances. Arrows show exposure initiation and duration. Based on [177-179] and papers I and II.](image)

**Figure 8.** Mechanistic studies of PBDE-induced neurodevelopmental disturbances. Arrows show exposure initiation and duration. Based on [177-179] and papers I and II.

The experimental in vivo studies on developmental neurotoxicity in this thesis are focused on three brain parts: hippocampus, striatum and cerebral cortex. There are several reasons for this. First of all, the main functional phenotype of PBDE exposure is a change in behavior. Hippocampus, striatum and cerebral cortex are all involved in short- and long-term memory formation and the execution of behavior and are therefore relevant for enquiry of mechanistic effects of developmental PBDE exposure. Secondly, one of the main hypotheses at the start of this thesis regarding the functional effects of neonatal PBDE exposure revolved the developing cholinergic system. These brain parts may be considered relay points in the cholinergic system, and are therefore potentially interesting. Thirdly, they are prominent and well-defined brain areas in the developing brain and thus can be identified and dissected with precision and in a reproducible fashion. Using an
approach aiming at identifying differences in protein expression, where the differences may be functionally significant, but relatively small on a quantitative scale, the absolute requirement for reproducibility in sample preparation can never be overstated.

In all our proteomics studies we have consistently used a 24 hours exposure regime. The underlying reason was that we considered 24 hours to be a sufficient time for exposure-induced changes on the proteome. Using a shorter exposure time may not only induce practical constraint, but also we could end up analyzing acute-toxic effects or PTMs, which was not the main focus. However, in paper IV we added a one hour exposure group with the avowed purpose to look for PTM changes.

In 2D-DIGE, 150 μg (3x50 μg) of CyDye labeled proteins are separated and analyzed. To make sure that enough proteins are available for subsequent MS analysis, preparative gels are often prepared containing at least 300 μg and up to 1 mg of proteins. This procedure induces a lot of extra-work, both running more gels and for spot-matching between analytical and preparative gels. In addition, the spot-matching also generates a potential source of error. In paper I, we solved this by adding extra, unlabelled proteins to one of the analytical gels in the experiment, thus eliminating this error source. This increased protein load did not influence the spot pattern, which was highly similar to the other gels. In addition, no effects were found in the subsequent data analysis, why we have used this procedure throughout. The only exception is paper III, where it was used but where we also prepared preparative gels which were matched to the analytical gels.

Hippocampus

The central importance of the hippocampus in learning and memory formation is highly recognized. The PBDE-99 induced effects on learning and memory dependent behaviors are therefore highly interesting. In paper I we wanted to analyze the effect in the neonatal hippocampus of 24 hours exposure to PBDE-99. The proteins were separated using a pH interval of 4-7. Using a cut-off level of 1.2-fold up- or down-regulation (corresponding to log₂ < 0.263) we found 56 differentially expressed proteins (p<0.01). Of these 56 proteins, 10 were successfully identified using MALDI-TOF MS (paper I, table I).

Of the 10 identified differentially expressed protein species in the hippocampus, three of them (α-Enolase, Atp5b and Idh3a) have previously been associated to PKC-ε complexes. Thus, being either a cause or consequence of PBDE-99 induced cellular effects; it seems as if PKC-signaling is a proximate target. The importance of PKC signaling in the modulation of motor behavior as well as in learning and memory is recognized [180, 181]. However, and importantly, the PKC association is indirect and the data provides no direct evidence of PKC involvement. Nevertheless, PKC has been
implicated as a target for other environmentally relevant xenobiotics, such as dioxins [182, 183], PCBs [180, 184-186] and more recently for other PBDE congeners [187, 188]. Thus, effects on PKC isoforms are by no means specific for PBDE-99 neurotoxicity but are rather a consequence of perturbing normal cell functions in the cell, such as calcium signaling. The effects on the expression of the multifunctional protein GRP75 (Mortalin) are interesting but difficult to comprehend. The function of Mortalin is dependent on subcellular location. Interaction studies have suggested that Mortalin may be involved in cell survival, control of proliferation and stress response [189]. More recently, this protein has been implicated in Alzheimer’s disease and Parkinson’s disease, with proteomics studies consistently identifying oxidatively damaged Mortalin as a potential biomarker [189, 190]. One α-Synuclein isoform was found to be differently expressed after PBDE-99 exposure (up ~40%). α-Synuclein is a presynaptically enriched protein that reaches a transient expression top around PND14 in the developing rat cortex and hippocampus [191]. There is a general increase in total brain α-Synuclein isoform expression between the first and the eighth week of life [192]. However, although α-Synuclein is of interest in this context due to its role in dopaminergic and glutamatergic signaling and hippocampal synaptic plasticity [193-195], the significance of this finding remains elusive.

**Striatum**

It is generally held that striatum and hippocampus provide competing frames of reference for action selection. While the dorsal/lateral striatum is more important for learning and choosing actions in body centered coordinates [196], the hippocampus is more important for remembering particular spatial locations defined by external cues [197]. Such experiments have also shown that hippocampal dependent strategies tend to dominate early in learning, while dorsal/lateral striatal-dependent strategies progressively dominate in late stages of learning [198]. The second part of paper I was focused on effects of PBDE-99 in the developing striatum. The same pH interval was used for the separation in the first dimension (pH 4-7), and the same criteria was used for cut-off (1.2-fold up- or down-regulation). 40 differentially regulated proteins were found in the striatum 24 hours after PBDE-99 exposure of which 9 spots were identified (paper I, table I).

Among the identified differentially expressed proteins in striatum, were isoforms of Gap43 and Stathmin. The two Gap43 isoforms were both upregulated by 25-31% and confirmed by immunoblotting using antibodies against Gap43. Three isoforms of Stathmin were found to be significantly downregulated (21-30%).

Stathmin is a cytosolic phosphoprotein that regulates the microtubule system and dendritic arborization [199]. The expression of Stathmin is closely correlated with neurite outgrowth but the expression is drastically reduced when
the brain cells have matured and the synapses have formed [200, 201]. The highly expressed Stathmin isoforms commonly detected in two-dimensional electrophoresis tend to become downregulated between PND7 and PND56 [192]. The upregulation of Gap43 and down regulation of Stathmin may seem contradictory considering their functional roles. Gap43 upregulation indicates increased neurite sprouting whereas Stathmin down regulation implies completed cell maturation and finished neurite sprouting. One solution to this contradiction may be that one or both proteins are posttranslationally modified potentially at multiple sites. So far, studies have identified ten different phosphorylation sites in Stathmin [202]. Two-dimensional electrophoresis (2-DE) studies have detected at least 16 different Stathmin isoforms after manipulation of only four of the phosphosites [203].

Mouse Gap43 has two cysteine palmitoylation sites and at least 13 different phosphorylation sites¹ [204]. Two-dimensional electrophoresis usually visualizes four Gap43 isoforms [205]. Different combinations of modifications are likely to give different functions, making assumptions about functional significance of up and down regulation of uncharacterized protein isoforms very problematic. Adding to the PKC discussion of the hippocampus section, both Stathmin and Gap43 are substrates for PKC isozymes. Both Stathmin and Gap43 are primarily found in neuronal lipid rafts [206, 207], to which PKC isozymes also translocate after activation.

Cerebral cortex

Paper II was the first study where we had the capacity to identify a larger amount of differentially expressed proteins. We also pre-fractionated the proteins into a soluble (cytosol) and less-soluble (membrane) fraction to increase resolution. 111 differentially expressed proteins were found in the cerebral cortex 24 hours after neonatal (PND10) PBDE-99 exposure, of which 73 had a unique identity (i.e. not an isoform of another protein in the study).

Using the DAVID database [208] we performed a functional annotation of all the identified proteins in the dataset, in which we identified three enriched subsets of proteins; cytoskeletal proteins, proteins related to oxidative stress and mitochondrial proteins.

An interesting finding in this paper was that almost one third (29%) of the differentially expressed and identified proteins are structurally or functionally associated to the cytoskeleton. The functional significance of this cytoskeletal effect is difficult to pin-point, but with such dramatic effects on cytoskeletal proteins, major disruptions in cell morphology would be expected. However, there is no support in the scientific literature of morphological effects on neural cells in the brain after in vivo PBDE exposure. Con-

¹ Information provided by PhosphoSitePlus at www.phosphosite.org
sidering that several of the affected proteins (Fatty acid-binding protein/E-FABP, Gap43, Tau, Uchl1) are associated with neurite sprouting [209-211], a process that is dependent on a functional cytoskeletal organization [212], it is possible that the processes involved in acquiring a functional neurite tree are affected.

The E-FABP is highly expressed in neurons during neurogenesis, particularly in the prenatal (GD17.5) and postnatal periods (PND5-PND10) in rats [213]. It is known to be co-expressed with Gap43 during axonal growth [209]. Two isoforms of Gap43 were up-regulated by 38-42% in the cerebral cortex after exposure. The up-regulation, which was confirmed by Western blot, is in accordance with the effect seen in striatum, but not hippocampus.

Gap43 and Gap43-like proteins (Brain acid soluble protein 1/NAP-22, Marcks) link the Actin cytoskeleton to membrane domains enriched in cholesterol and sphingolipids; lipid rafts or detergent-resistant membrane (DRM) fractions. These fractions are enriched in glycosylphosphatidylinositol (GPI)-anchored proteins and transmembrane receptor proteins [204, 214]. Interest in lipid rafts arises from observations that some membrane proteins appear to partition preferentially to raft domains, and may even require this environment for their biological activity [207]. DRM fractions of cerebellar granule neurons (CGNs) are enriched in Gap43 and phosphorylated Gap43 but not in MARCKS or PKC proteins [206]. PKC activation or glutamate stimulation in CGNs both lead to the enrichment of PKC protein in the DRM. After translocation of PKC to DRM, Gap43 is phosphorylated within this membrane compartment and then moves outside to other membrane zones [206]. Gap43 has recently been analyzed after PBDE-209 induced developmental neurotoxicity, after exposure on PND3 (Figure 8). Interestingly, contrary to our result they see a down-regulation of Gap43 in cerebral cortex 7 days after exposure [179], which may be explained by differences in sample preparation. The sample preparation used in that study (extraction using triton X-100 at cold temperature) isolates the rafts in the resulting pellet. However, since they have analyzed the supernatant, most of the Gap43 will be lost [204, 206].

Besides Gap43, two other (CRMP-3, Brain acid soluble protein 1/NAP-22) differentially expressed lipid raft proteins were found in our study, perhaps indicating an effect on lipid-raft mediated signaling. More specific analyses of raft-associated proteins are needed to make any such conclusions.

Cultured cerebral cortex cells

Cell viability

Due to the apparent effects on multiple mitochondrial and oxidative phosphorylation proteins in the cortex and previous effects on proteins associated
with stress in the striatum and hippocampus, a study was performed to de-
duce the effects of PBDE-99 on cell viability.

The \textit{in vivo} mouse model is based on PND 10 mice, why the most rele-
vant \textit{in vitro} cell model perhaps would be to use cultured cells from PND 10 mouse cerebral cortex. However, it is not possible to make primary cortex (or hippocampus) cultures from PND 10 mouse cells: the cells do not sur-
vive. It is possible to culture cortex cells from the PND1 mouse brain, but
soon after that time point the cells lose their plasticity and adaptability and
cannot endure culturing. Cerebellar granule neurons (CGNs), however, can
be cultured after isolation from PND7 animals or even later. The underlying
reason is the bi-phasic development of the cerebellum, where Purkinje cells
develop early (GD 13-15 in the rat) while granule cells are generated postna-
tally (PND 4-19) [215].

We thus cultured primary cortical cells from GD17 rat fetuses (\textit{Figure 1D}). 24 hours after culturing they were exposed to PBDE-99 in different
concentrations (3, 10 or 30 μM) for 24 hours. Cell viability (assessed with
the trypan blue assay) was significantly decreased after 24 h of PBDE-99
exposure but only at the highest concentration (30 μM). In addition, we
found no effects on viability after 3 μM or 10 μM PBDE-99 exposures for
72 h, suggesting that these concentrations are not high enough to cause ne-
crosis during the first 72 h in cell culture. We found no evidence of increased
caspase-3 activity. An apoptotic cell morphology in the absence of increased
caspase-3 activity has been found in cultured cerebellar granule cells ex-
posed to a commercial PBDE mixture [216] which makes it unclear to what
extent PBDE-99-induced cell death is due to apoptosis.

To test the hypothesis of PBDE-99-induced effects on neurite sprouting,
we used the same cell culture approach as above, but double-stained the cells
with Tubulin-βIII and the cell nucleus stain DAPI (\textit{Figure 9}). The images
were analyzed using fractal analysis [217, 218] to deduce effects on neurite
sprouting and arborization. Although we could see a positive trend on neu-
rite sprouting after 24 hours exposure to the lowest concentration (3 μM), no
statistically significant effects were found, except for the reduction of neurite
sprouting seen at the highest concentration (30 μM) which was associated
with cell death (\textit{Figure 9}).
Figure 9. PBDE-99 effects in GD17 (A, B and D) and GD21 (C, E and F) rat cerebral cortex cells cultured for 24 hours. A. Cultured immunolabeled cells. Green cells are Tubulin-βIII positive, while blue cells are DAPI positive. B. Neurite sprouting measured with the fractal analysis method. 30 μM PBDE-99 significantly reduced the D-value, suggesting reduced sprouting. (*p<0.05). C. Effects on Gap43 mRNA and protein expression. 0.3 μM PBDE-99 significantly upregulated the mRNA expression (p<0.01) and 30 μM significantly downregulated the mRNA expression (p<0.05). Error bars display standard error of the mean (SEM). No apparent effects on Gap43 protein expression except a downregulation at the 30 μM concentration. 0.3 and 3 μM PBDE-99 clearly upregulated the expression of phosphorylated Gap43. D and E. Cell viability measured using the trypan blue assay (*p<0.05, **p<0.01). F. STEM derived profile for the protein spots in paper IV. 13% of the protein spots display an expression profile where low and high PBDE-99 concentrations have opposite effects on protein expression (“reverse profiles”).
The experiment was repeated using 72 hours and 7 days exposure to PBDE-99, and although the trend was still there for the 3 μM concentration, no significant effect was seen (data not shown). The approach we have used to evaluate the effects on neurite sprouting is insensitive for specific effects on axonal or dendrite sprouting. A more direct approach for neurite sprouting (using e.g. a MAP2 antibody for dendrite specific sprouting and a Tau antibody for axon sprouting [219]) will perhaps yield more conclusive results.

The cell viability test was subsequently performed using cerebral cortex cells from GD21 rat pups (i.e. 4 days later in gestation) (Figure 1). Reduced viability was noted already after 10 μM PBDE-99 exposure (Figure 9), indicating that the GD21 cells are more sensitive than the previously reported GD17 cells, perhaps reflecting the difference in cell composition between the two time points. In preparative studies before paper II, it was noted that culturing GD17 cerebral cortex cells (neuroblasts) for 24 hours gives mainly neurons, while the same culture conditions for GD21 cells generates a co-culture with neurons and GFAP-positive glia cells. This reflects the pattern of cerebral cortex neuroblast differentiation (Figure 1).

**In vitro Proteomics**

*In vitro* systems with neuronal and astroglial cell preparations are increasingly used for mechanistic studies in neurobiology and medicine. Paper IV is an *in vitro* proteomics approach which was performed to establish and evaluate an *in vitro* neurotoxicity model, but also to make *in vivo*-in *vitro* comparisons with the cerebral cortex study. The samples for 2D-DIGE were derived from rat primary cortical cells isolated at GD 22 (Figure 1B, D), that were cultured for 24 hours prior to exposure. Besides two control groups (control and DMSO-control), three exposure groups were used (3 μM, 10 μM, 30 μM). The cells were exposed for 24 hours prior to protein extraction. It is estimated that the PBDE-99 concentration in the brain after in *vivo* exposure is in the same molar order of magnitude as the doses used in the in *vitro* study [220].

In addition to these exposure groups, a 1 hour exposure group (30 μM) was matched to the other set. This resulted in two data sets, one smaller that encompasses both “24h samples” and the “1h samples” (an estimated total of 345 spots), and one larger “24h samples” only that represents the dose response data (an estimated total of 816 spots). A total of 292 proteins were identified of which 156 were found in the smaller data set.

STEM profile analysis and PCA-analysis independently identified dramatic effects in the 30 μM exposure group (Figure 7). Subsequent cell viability analysis resulted in a significantly increased (p<0.01) cell death after 30 μM exposure, suggesting that the major effects on the proteome may be
at least partly due to cell death. Cell death was also increased (p<0.05) in the 10 μM exposure group (Paper III, Figure 1).

The majority of identified proteins shared between 10 μM and 30 μM were affected in the same pattern of up- or down regulation (26 out of 28 spots had the same pattern). Only 22 out of 41 spots had the same pattern when 3 and 30 μM were compared. Thus, when a protein is up-regulated at the 10 μM level, it is likely that the protein is up-regulated also at the 30 μM level. However, if the protein is up-regulated at the 3 μM level, it is just as likely that the protein is down-regulated at the 30 μM level.

Interestingly, 52% (25 proteins) of the proteins affected and identified at 3 μM are cytoskeleton related according to the DAVID database [208] while only 23% (10 proteins) and 37% (87 proteins) meet the same criterion in the 10 μM and 30 μM samples respectively (Figure 10). This is in accordance with the effect seen in cerebral cortex in vivo [221] where dramatic effects were seen on cytoskeletal proteins after PBDE-99 exposure.

![Figure 10. Annotation of the identified proteins in paper IV. Proportion of the identified proteins in the exposure groups that are annotated as cytoskeletal (annotation cluster 9 in the functional annotation using “cytoskeleton organization and biogenesis”, “cytoskeletal protein binding” and “cytoskeleton”) and mitochondrial using “mitochondrion” in the DAVID database [208].](image)

33 proteins were identical between the in vivo cerebral cortex study and the in vitro study, of which 26 were significantly (p<0.05) differentially expressed in vivo. β-Actin (ACTB), one out of five detected in vitro isoforms) and Dihydropyrimidinase-related protein 3 (Dpyl3), one out of eight detected in vitro isoforms (Figure 7) were especially interesting since they were significantly affected in vivo and in the in vitro concentration and time response studies.

To determine whether there was a correlation between PBDE-99-induced changes in mRNA and protein expression, the protein identities were cross-referenced against mouse MEEBO microarray results from 24 h 3 μM and
30 μM exposures. Only expression data from probes >95% identical in sequence between rat and mouse, which after sequence comparison and filtering resulted in 16840 probes for the 3 μM study and 19766 for the 30 μM study. We only detected a weak correlation between mRNA and protein changes in response to 3 μM treatment, whereas the more toxic high dose resulted in mRNA and protein changes that showed a greater correlation (paper IV, Figure 4).

**In vivo-In vitro comparison**

The relevance of *in vitro* findings for *in vivo* effects is frequently debated. What is generally agreed upon, however, is that *in vitro* findings can be used to design *in vivo* studies and can be useful in determining the mechanisms by which a toxicant might affect a certain cellular function or developmental process [222]. However, cell culture studies of neurotoxicity are by themselves abstract, and must be interpreted in the context of biological complexity. In addition, they contribute the greatest insight to biology when they are performed in complement with *in vivo* experiments.

In paper IV an *in vitro* approach was used, based on cultured cerebral cortex cells which were exposed to PBDE-99 using a concentration and exposure time with relevance for our previous *in vivo* study. Thus, by identifying the proteins that are differently regulated after *in vivo* and *in vitro* exposure, it may be possible to find proteins directly affected by PBDE-99 rather than via indirect effects (paper IV, Figure 4). The proteins were matched based on protein identity only, and not on spot matching across experiments. A total of 33 unique proteins were found in paper IV that were affected both *in vivo* and *in vitro*, which may be used for further inquiry of direct effects of PBDE-99 on cortex cells.

*Dpyl3*

Of these proteins, Dpyl 3 was found to be differently regulated already after 1 hour exposure to PBDE-99 *in vitro*. With the preconceived opinion that most proteins, except for immediate early proteins such as c-Fos and c-Myc, require more than one hour for *de novo* protein synthesis, this suggests that the change in expression seen for this protein after one hour is based on changes in PTMs. This is further reinforced by the finding that no changes were found for the corresponding gene on the mRNA level. In addition, by using the Pro-Q Diamond gel stain which specifically stains phosphorylated proteins we showed that the Dpyl 3 isoform was phosphorylated. Even if our initial MS-based experiments for detailed characterisation of the specific PTM have failed, this is an example of how to use 2D-DIGE for finding isoformspecific effects of treatments in biological systems.
**Gap43**

Gap43 was upregulated in both *in vivo* mouse striatum and cerebral cortex 24 hours after neonatal PBDE-99 exposure. Although it did not show up on our 2D-DIGE gels in the *in vitro* study (paper IV), western blot was used to analyze the effects on protein expression. RT-PCR was used to deduce the effects on mRNA levels. 0.3 μM PBDE-99 significantly increased the mRNA expression, while no clear effects were seen on total protein expression at any of the tested concentrations. However, both 0.3 and 3 μM PBDE-99 apparently increased the expression of phosphorylated Gap43 compared to the other treatment groups. The fact that very low doses that are not correlated with cytotoxicity affects both mRNA expression and PTM status of Gap43 (Figure 9) suggests that PBDE-99 may affect regulatory pathways controlling Gap43 expression. It would be interesting to compare the low dose effects of different PBDE congeners on Gap43 expression in vitro, since PBDE-99 and PBDE-209 show potentially conflicting effects on Gap43 expression in *vivo* [179].

The studies of PBDE-99 induced toxicity in cultured cerebral cortex cells collectively indicate that low concentrations of the substance in some respects may have opposite effects to high concentrations. The “reverse profiles” in the STEM derived picture (Figure 9F) in combination with the low-concentration effects on Gap43 expression and the viability studies indicates that low concentrations of PBDE-99 may have stimulatory effects on cell differentiation and survival. If this holds true also after detailed, scrutinizing *in vivo* studies, the effects on behavior seen after developmental PBDE-99 exposure can be related to a miswiring of the adult rodent brain.
Concluding remarks

The work presented in this thesis has brought about new hypotheses for the disruptive effects of PBDE-99 on neonatal brain development. In addition, since a considerable part of the thesis is focused on proteome-wide changes, it provides new ideas regarding how to perform, present and evaluate proteomics experiments.

It is interesting to note the differences in response to PBDE-99 between the hippocampus, striatum and cortex. Although the complement of expressed proteins is very similar for all the brain parts, the responses to PBDE-99 are apparently discrete. This likely reflects the underlying heterogeneity between different brain parts and cell populations, and the fact that the brain parts examined are in different developmental stages. For example, while the major part of the neurogenesis is finished in the striatum at the time of exposure, the hippocampus is at the height of dentate granule cell neurogenesis (Figure 1). This heterogeneity with respect to brain region may not encourage in vitro initiatives where effects on proteins in tissue specific cell culture models are used for neuron wide generalizations.

The major effects seen in the in vivo cerebral cortex, but also after low-dose exposure in vitro are interesting. Profound cytoskeletal disruptions are not expected without effects on cellular morphology. No evidence was found for macroscopic effects on cell morphology in vitro and there is no scientific evidence of in vivo neural structural effects of PBDE-99 exposure in the literature.

The seminal finding in paper I, which has influenced our current thinking about PBDE-99 neurotoxicity, was that the growth- and plasticity-associated protein Gap43, a protein with a key role in guiding axon growth and modulating synapse formation, was upregulated in striatum. In the striatum we also found decreased levels of Stathmin. Similar changes (i.e. up-regulation of Gap43 and down-regulation of Stathmin) have been observed in neurodegenerative disorders such as Alzheimer’s disease [223, 224]. Interestingly, Gap43 was also up-regulated by PBDE-99 in the cerebral cortex (paper II) in association with down-regulation of the functionally related brain acid soluble protein 1 (Basp1/CAP-23/NAP-22). Normally, both of these proteins are highly expressed in neuronal growth cones during CNS synaptogenesis, after which they become down regulated, except in some specific brain regions where plasticity is retained [225, 226]. Even if the functional significance of
the effects on Gap43 and NAP-22 remain elusive, a possible scenario is that they lead to a miswiring of circuits critical for memory formation.

This thesis work shows the usefulness of gel-based proteomics techniques in toxicology. Armed with knowledge that a protein has changed in amount or composition, it is possible to form a hypothesis about the cause and consequences of such a change. However, without the development of corroborative information about mechanisms, this will be limited to a rather speculative conjecture. This is particularly true if the number of identified proteins is limited, as in paper I. An alternative approach is focusing on the associations. Without understanding the biological mechanisms, it is possible to associate certain protein changes with a biological or functional outcome. The large-scale gene studies (microarrays) have developed along these lines, and it is reasonable to assume that this is also where proteomics is heading. However, to fully make that leap it is necessary to generate protein based approaches for GO-like analyses that also take into account the PTMs. The continued development of GO-based tools like DEPPS, in combination with appreciation of the biology “hidden” in the PTM patterns will even further add to the applicability of the proteomics technology base.

A 24 hours exposure regime has been consistently used, since that time period was considered to be sufficient for de novo synthesis of proteins while not merely displaying acute toxicity. Although consistency has its merits, this will give a limited insight into the dynamics of protein expression. Any effect, let be indirect, compensatory or a rebound effect that appear later than 24 hours after exposure have not be considered. In addition, the in vivo model consistently used PBDE-99 exposure on PND 10, which is at the peak of the BGS. As a consequence, we have no means of comparing the effects after exposure to PBDE-99 outside this window of sensitivity.

The aim of paper III was to deduce the molecular background or reason for the dyskinesias which frequently develop during pharmacological treatment of Parkinson’s disease with L-Dopa. An interesting finding was that there were significant effects on protein levels already one hour after L-Dopa treatment (Figure 5). This is fascinating since for the vast majority of proteins this time is not sufficient for de novo protein synthesis, suggesting that the effects may be due to PTMs. Therefore in paper IV we decided to include an acute exposure group with the rationale being that this would help us identify proteins where the change in protein expression after 24 hours could be related to PTMs rather than de novo synthesis. We also used it to see if we could identify any PTMs possibly underlying these effects. However, from a more general perspective, I think it would be wise to always include a short (one hour or less) exposure group in 2-DE experiments to control for PTM effects rather than studying effects on protein expression alone.
Future perspectives

This thesis gives directions for further studies in the field, especially regarding potential markers of effect and subproteomes of interest for more detailed enquiry with respect to PBDE-99 induced neurotoxicity.

The rationale used for the in vivo studies of PBDE-99 induced developmental disturbances was to take a snapshot of the molecular processes taking place 24 hours after PBDE-99 exposure. Although this approach has its merits, and has provided an overview of the early effects on protein expression in three discrete brain regions, a number of improvements are conceivable for future studies in the field.

The expression of a protein is usually transient and reflects the need of that particular protein in the current state of the cell. Accordingly, changes in cellular environment (such as those induced by a toxicant) will change the metabolic or in other ways functional needs of the cell, which concomitant changes in protein expression. The preconception used in this thesis is that the early changes in protein expression induced by PBDE-99 may give rise to changes in availability of e.g. trophic signals or structural proteins that will redirect the normal developmental trajectories, and ultimately emerge as a functional impairment of the adult brain. Behavioral studies of rodents exposed to PBDEs have shown that the sensitivity period is passed at PND 19 [72], suggesting that any effects on protein expression induced after that time period are not likely to induce or reflect changes associated with a change in adult behavioral phenotype. Accordingly, comparing the effects induced within and outside the window of sensitivity can give better information about whether a particular change in the expression of a protein or a group of proteins is likely to be associated with downstream effects on adult brain function.

This difference between sensitive (mouse PND10) and not (or apparently less) sensitive (mouse PND19) perhaps reflects that while the mature brain compensates to accommodate changes in the environment, the immature brain adapts by incorporating the environmental information permanently into the mature structure and function. Although proteins are the true messengers of biology and in the end will manifest the biological effects of the exposure, the “vector” carrying the irreversible changes linking the exposure in the developing neonatal brain to the adult behavioral phenotype may be of a different nature.
The term imprinting describes the long-lasting effects that endure well after the removal of the originating agent [227]. On a molecular level, imprinting of epigenetic factors may be defined as those that have a meiotically and mitotically heritable influence on the chromosome or gene function without being coded in the DNA sequence itself [228]. The process works by either DNA methylation or by histone methylation or acetylation and regulates gene expression by blocking transcription of regulatory factors, repressing gene expression or regulating transcription activity [229]. Recent studies have shown that mouse dams that display a strong nurturing behavior towards their pups produce lasting alterations in the patterns of DNA methylation in the CNS of their pups, which persist into adulthood [230-233]. These changes in DNA structure resulted in decreased anxiety and a strong maternal nurturing instinct in the adult offspring. This persistence of neonatally acquired patterns of DNA methylation in the adult CNS is consistent with the hypothesis that epigenetic mechanisms contribute to lasting cellular effects, in the form of cellular memory in the CNS [228]. In a wider perspective it suggests that a critical environmental exposure can alter the progression of epigenetic programming during neonatal development, and that an environmental exposure during this critical period can result in epigenetic and therefore phenotypic differences later in life. Thus, exploring the effects of PBDE-99 on the hippocampal and cortical epigenome seems like an attractive path for further exploration of the mechanistic basis of PBDE-99 induced behavioral disturbances. In addition, histone modifications as such are not permanent. Rather, recent studies have shown that neonatal epigenetic programming is reversible by pharmacological treatment in the adult [230], thus reversing the effect of maternal care on behavior. It remains to be seen if a similar approach is applicable for PBDE-induced developmental neurotoxicity.

Although 2-DE has its worth, including the ability to separate and visualize protein isoforms, other preferably MS-based techniques are needed to give a more thorough view of the proteome and epigenome. In particular, it would be useful to get more spatial information about the protein expression, in combination with quantitative estimates. MALDI imaging mass spectrometry is a technique that is suitable for such studies (Figure 11).
Svensk sammanfattning

Hjärnans utveckling är hos däggdjur en lång, integrerad serie händelser som från ett fåtal neuronala prekursorer skapar en fullt utvecklad hjärna med en central roll för organismen. Neurotoxikologisk forskning har påvisat ett antal känslighetsfönster under vilka den normala utvecklingen är särskilt känslig för störningar. En sådan ”kritisk period” passeras i nyföddhetsperioden hos människa då hjärnan genomgår sin kraftigaste tillväxt.

Studier i gnagare under denna utvecklingsfas har påvisat en ökad känslighet för ett betydande antal xenobiotika, såväl läkemedel (ketamin, propofol) och rekreationsdroger (etanol, nikotin) som miljögifter (PCB, DDT, PBDE). Individer som exponerats under denna fas upptas ett onormalt beteende, med såväl hyperaktivitet som minnes- och inlärningsströningar, som vuxna. Mycket lite är känt om de underliggande orsakerna till dessa beteendestörningar, på såväl fundamentalt övergripande som mer mekanistisk nivå.


Mer övergripande påvisar avhandlingen värdet av gel-baserade proteommetoder för neurotoxikologisk forskning. Genom att utvärdera effekter på proteom snarare än protein-nivå ges möjligheter till att utvärdera globala effekter i ett biologiskt system. Detta ger i sin tur mer konfidens i de biologiska slutsatserna.
Sammanfattningsvis visar denna avhandling att de störningar i hjärnans utveckling som uppkommer genom neonatal exponering för PBDE-99 kan studeras på proteinnivå. I flera fall är effekterna isoform-specifika, vilket understryker behovet av att bättre annotera och förstå de biologiska effekterna av posttranslationella modifieringar.
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