Department of Medicine, Karolinska Institutet, Stockholm, Sweden

Cell Interactions in the CNS and their Consequences for Neuronal Apoptosis

Akademisk avhandling

som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras på engelska språket i Leksellsalen, Eugeniahemmet, Karolinska Universitetssjukhuset Solna, torsdagen den 12:e maj 2005 klockan 9.30

M I K A E L A B E R G L U N D

S T O C K H O L M 2 0 0 5

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Cover Illustration:

SH-SY5Y neuroblastoma cells cultured for 72h, followed by fluorescence labeling of nuclei (Hoechst) and filament (βIII-Tubulin). The image has been converted to black/white and inverted in Adobe® Photoshop®CS.

Published and printed by Larserics Digital Print AB, Bromma, Sweden

ISBN: 91-7140-222-5

"I may not have gone where I intended to go,
but I think I have ended up where I intended to be"

DOUGLAS ADAMS

ABSTRACT

The central nervous system consists of an intricate network of neurons and glial cells that depend on reciprocal communication to develop and maintain the vital functions of the brain. If the balance is disturbed by injury or disease, two cell types in particular, microglia and astrocytes, respond by producing factors that help combat the infection or delimit the affected area. The microglial response can be beneficial promoting neuronal survival, however it can also cause harm to the surrounding cells. To further investigate this balance, and subsequently for studies of neuronal apoptosis, different cell culture techniques spanning from complex primary co-cultures to one-dimensional cell line studies were applied.

Fetal mouse brain cells were dissociated and cultured under gyratory rotation to form three-dimensional aggregates allowing for cell-cell contact between neurons and glia. The aggregates were characterized in respect to cell contents, viability, expression of neural transmitters, and response to mitogens. The aggregates presented a glia:neuron ratio of 1:20 at day 4 changing to 1:4 until day 16, which correlates to the increased postnatal proliferation of glia *in vivo*. Both neurons and astrocytes matured during the culture period in regard to increased expression of synaptic proteins and GFAP respectively. Treatment of the aggregates with EGF induced proliferation of nestin positive cells that were designated as neural precursor cells due to their ability to renew, propagate, and differentiate into neurons and macroglia. Neural precursor cells were maintained in the aggregates for more than two months in culture. This aggregate culture system provides a useful tool for *in vivo*-like *in vitro* studies combining cellular complexity with a controlled environment.

Studies of neuron-microglia interactions were initiated using a cell line-based model system where SH-SY5Y neuroblastoma cells were co-cultured with THP-1 monocytes. As a means to morphologically detect and quantify potential monocyte-induced neuronal apoptosis, an automated fluorescence imaging system, ImageXpress (IX), was utilized. To validate the system, SH-SY5Y cell apoptosis was induced by rotenone. The treatment resulted in chromatin condensation and nucleus size reduction, parameters that were used in combination with nuclear intensity to distinguish apoptotic cells from normal cells. The IX system proved to be a suitable tool for detecting rotenone-induced neuronal apoptosis. However, activated THP-1 cells did not induce apoptosis in SH-SY5Y cells, as detected with the IX system. These results were confirmed by an ELISA-based apoptosis assay. Subsequently, we cultured SH-SY5Y cells in the presence of medium that had been conditioned (CM) by activated THP-1 cells for 24h, hence containing higher levels of potentially neuromodulatory factors than could be achieved in the co-culture experiments. We observed that CM from LPS and LPS+IFN-γ stimulated monocytes induced neuronal apoptosis in a dose dependent manner. The effect of LPS+IFN-γ stimulation was partially mediated by TNF-α. We concluded that unphysiological ratios of monocytes to neurons are needed to induce neurotoxicity in this system.

Finally, we wanted to investigate whether microglia could rescue damaged neurons from apoptosis, or possibly delay the course. To map a window where apoptosis was initiated, but not committed, SH-SY5Y cells were treated with doses of UV irradiation. We found that activation of JNK and c-Jun was induced early after insult, followed by cytochrome c (cyt c) translocation, cleavage of caspase-3, and ensuing apoptosis. JNK-inhibition induced a 60% reduction in c-Jun phosphorylation (Ser63/Ser73), but did not prevent mitochondrial cyt c release or apoptosis, indicting that JNK is not essential for UV-induced apoptosis in SH-SY5Y cells. We conclude that UV irradiation is a complex stress that may induce multiple mechanisms contributing to apoptosis.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their roman numbers (I-IV):

I. C. Mikaela D. Berglund*, Johan Aarum*, Tomas Hökfelt, Jens R. Nyengaard, Samantha L. Budd Haeberlein, Jan Näslund, Kristian Sandberg, Mats A. A. Persson. **Characterization of long term mouse brain aggregating cultures: evidence for maintenance of neural precursor cells.**
J. Comp. Neurol. 474(2):246-260 (2004).

II. C. Mikaela D. Berglund, Ann-Cathrin Radesäter, Mats A. A. Persson, Samantha L. Budd Haeberlein. **Automated imaging as a means to measure apoptosis in human SH-SY5Y neuroblastoma cells.** *(Manuscript).*

III. C. Mikaela D. Berglund, Mats A. A. Persson, Samantha L. Budd Haeberlein. **Induction of human SH-SY5Y neuroblastoma cell death by stimulated THP-1 monocytes.**
(Manuscript).

IV. C. Mikaela D. Berglund, Ann-Cathrin Radesäter, Mats A. A. Persson, Samantha L. Budd Haeberlein. **UV-induced apoptosis in SH-SY5Y cells: contribution to apoptosis by JNK signaling and cytochrome c.**
J. Neurosci. Res. 78(4):580-589 (2004).

* These authors have equally contributed to the study.

ABBREVIATIONS

ab	antibody
AD	Alzheimer's disease
AIF	apoptosis inducing factor
ANOVA	analysis of variance
Apaf-1	apoptotic protease activating factor 1
ATP	adenosine triphosphate
BBB	blood brain barrier
Bcl-2	B-cell leukemia/lymphoma 2
Bcl-x_L	B-cell leukemia/lymphoma x Long (long isoform)
BDNF	brain derived growth factor
bFGF	basic fibroblast growth factor
Bid	BH3-interacting domain death agonist
BrdU	5-bromo 2-deoxyuridine
caspase	cysteinyl aspartate-specific proteinase
CDK	cyclin-dependent kinase
CGRP	calcitonin gene-related peptide
CM	conditioned medium
CMFDA	green 5-chloromethylfluorescein diacetate
CNPase	2, 3-cyclic nucleotide 3-phosphodiesterase
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CR3	complement receptor 3
cyt c	cytochrome c
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ED	embryonic day
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis(aminoethylether)-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated protein kinase
FADD	Fas death-domain protein
FJ	Fluoro-Jade
GFAP	glial fibrillary acidic protein
h	hour(s)
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
Iba1	ionized calcium binding adapter molecule 1
IFN	interferon
IL	interleukin
IX	ImageXpress
JNK	c-Jun N-terminal kinase
LDH	lactate dehydrogenase

LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MEKK	MAP/Erk kinase kinase
MG	microglia
MHC	major histocompatibility complex
MKK	mitogen-activated protein kinase kinase
MOG	myelin oligodendrocyte glycoprotein
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
NADH	nicotinamide adenine dinucleotide
NeuN	neuronal nuclei
NGF	neural growth factor
NO	nitric oxide
NPY	neuropeptide Y
NT	neurotrophin
PBS	phosphate buffered saline
p-c-Jun	phosphorylated c-Jun
PCNA	proliferating cell nuclear antigen
PD	Parkinson's disease
PFA	paraformaldehyde
p-JNK	phosphorylated c-Jun N-terminal kinase
PLP	myelin proteolipid protein
PNS	peripheral nervous system
PS	phosphatidylserine
ROS	reactive oxygen species
rpm	revolutions per minute
SAPK	stress activated protein kinase
SEM	standard error of the mean
Smac	second mitochondria-derived activator of caspase
tBid	truncated Bid
TGF	transforming growth factor
TH	tyrosine hydroxylase
TMT	trimethyltin
TNF-a	tumor necrosis factor a
TNFR1	tumor necrosis factor receptor 1
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling
UV	ultra violet
VIP	vasoactive intestinal polypeptide

Abbreviations used only once are described where they appear in the text.

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PREFACE

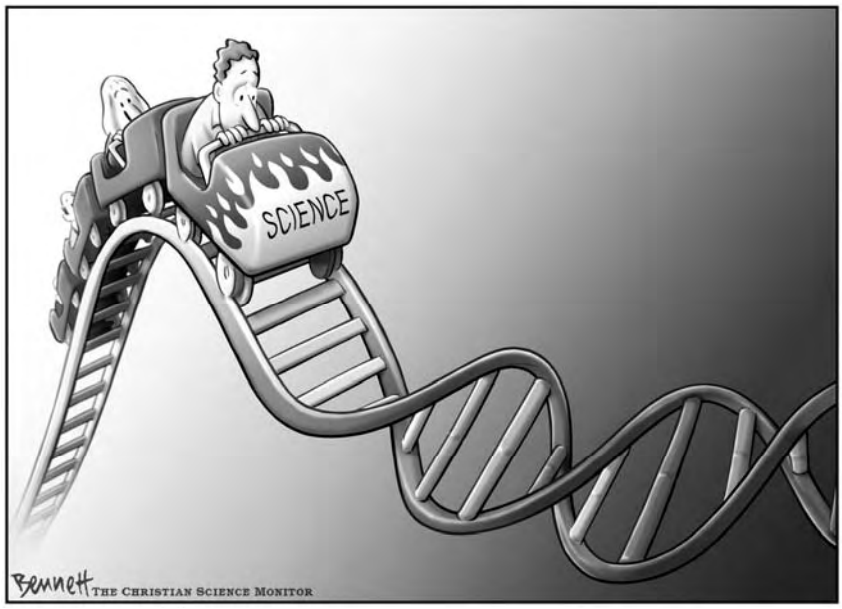
This thesis covers the main projects that I have worked with during the past 5 years. In retrospect, I have to admit that the straight road I saw ahead of me when I registered as a PhD-student rather quickly turned out to be a thrilling roller coaster. I have learned a lot on this ride, not only about science, but also about life and myself. My initial interest when I started my PhD was microglia (MG), a complex, brain resident cell type that possesses immunological features. One problem studying MG has been the lack of specific markers that distinguish them from their close relatives, the monocytes/macrophages. Our first aim was to search for an exclusive microglial marker using phage display, a method that was well established in the lab. Quite soon though, the roller coaster took an unexpected turn and headed towards setting up a complex cell culturing technique together with associates at AstraZeneca. After many long nights learning how to dissect cortex and hippocampus from tiny mice embryos, we managed to turn the dissociated brain tissue into "mini brains" or fetal mouse brain cell aggregates to be specific. Our characterization of the aggregates resulted in Paper I.

After spending three years on the aggregates, I took a moment to think over my project, aims, and PhD-studies in general. After discussions with supervisors, others, and myself I decided to focus on MG again. There is an intriguing balance between the neurotoxic and neuroprotective features of MG. I wanted to induce neuronal injury and study what effect the neurons had on MG and *vice versa*. As I worked out the injury model, I was introduced to the fields of apoptosis (cell death) and cell signaling, areas that I always found too complicated to get involved in. Despite this, or maybe because of this, it fascinated me. As my studies progressed, we encountered some interesting findings regarding UV-induced neuronal injury and the apoptotic pathways involved. Chronologically this resulted in my second publication that came to be Paper IV in this thesis.

In my two final projects, I had the opportunity to use the ImageXpress (IX) facility at AstraZeneca. A fluorescence microscope combined with an image based soft ware allowing for rapid imaging and analysis of cells in culture. I evaluated the use of this system as a means to detect neuronal apoptosis induced by the mitochondrial toxin rotenone. The results from these experiments were concluded in Paper II.

I continued by investigating monocytic effects on neurons in culture with emphasis on their proposed neurotoxic features. We found that neurons cultured in presence of conditioned media from stimulated monocytes underwent apoptosis. However, culturing the neurons in contact with the corresponding monocytes did not have the same effect. These studies resulted in Paper III.

Finally, it was time to write up my thesis, something I had looked forward to very much (although finding a common denominator connecting my four papers was a bit of a headache). The result is now in your hand with the title "Cell interactions in the CNS and their consequences for neuronal apoptosis". I have very much enjoyed writing it and I hope you will like the reading!



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INTRODUCTION

The nervous system is a complex communication network that permeates our entire body regulating functions as diverse as motility, perception, learning, and memory. The nervous system consists of the central nervous system (CNS), which includes the brain and spinal cord, and the peripheral nervous system (PNS) that provides the communication link between the CNS and the rest of the body. The PNS also reports signals from the environment back to the CNS. Thus the communication between the CNS and the PNS operates in two directions. The CNS can be divided into seven distinct functional regions specialized in performing different tasks (Figure 1). The spinal cord receives sensory signals from the skin, muscles, and joints via the PNS and mediates reflexes and movement. The upper part of the spinal cord passes into the medulla oblongata, which together with pons and the midbrain constitute the brain stem; the most primitive part of the CNS. This region is well conserved between different species and regulates vital functions such as breathing, digestion, cardiovascular control, and eye movement. Pons also mediates information about movement from the cerebral hemisphere to cerebellum, which modulates the force and range of these actions. In vertebrates, the cerebral hemisphere is highly developed and contains the cerebral cortex and hippocampus, which together are responsible for our cognitive abilities, sensory perception, learning, and memory. The diencephalon contains two structures, the thalamus, which works as a relay station directing information to different parts of the cerebral cortex and the hypothalamus that regulates autonomic and endocrine functions.

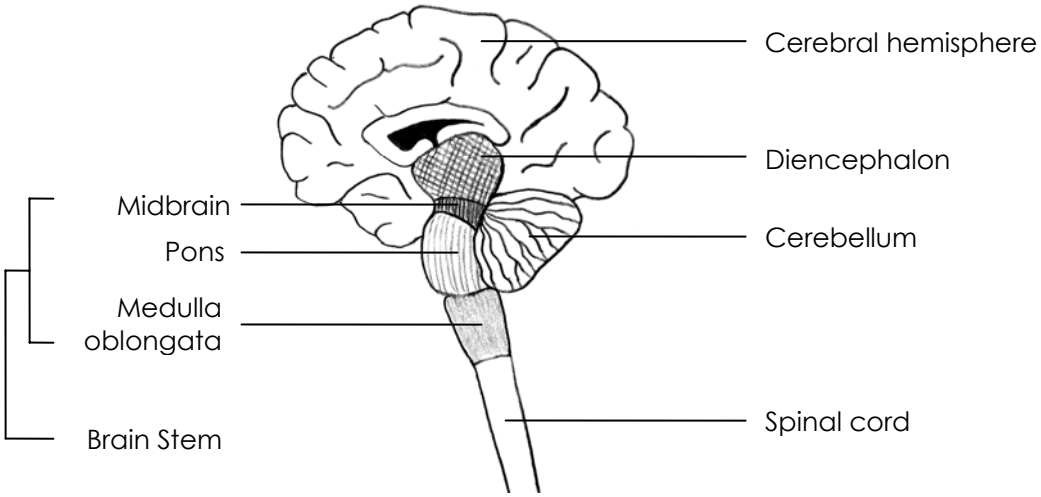

Figure 1: *Main structures of the CNS.*

NERVOUS SYSTEM DEVELOPMENT

The nervous system develops from the ectoderm, which together with the endoderm and mesoderm constitute the three cellular layers that ultimately give rise to the different organs of an organism (Jessell and Sanes, 2000). The formation of tissues and organs is regulated by differential expression of specific genes induced by factors released by surrounding cells. The ectoderm receives mesodermal signals in order to form the epidermal ectoderm, which later becomes the skin, and the neural plate that give rise to neuronal tissue. The neural plate folds into the neural tube where early regional divisions of the nervous system are formed. Within each region, progenitor cells develop into neurons and glia. The cell fate is controlled by the access to growth factors such as neural growth factor (NGF), ciliary neurotrophic factor (CNTF), and platelet-derived growth factor (PDGF) that promote the generation of neurons, astrocytes, and oligodendrocytes respectively (Jessell and Sanes, 2000; Johe et al., 1996). The presence of growth factors also helps to repress the cell death program that is present in all cells. If a cell fails to respond to growth factors, it undergoes programmed cell death, a process also called apoptosis. It has been estimated that during the embryonic and early postnatal development approximately 50% of the neurons formed by neurogenesis die by apoptosis (Ham et al., 2000; Oppenheim, 1991). The surviving cells migrate to their final destinations, differentiate and establish connections with surrounding cells. Here, failure to establish functional synapses is a cause for induction of apoptosis.

CELLS IN THE CENTRAL NERVOUS SYSTEM

Neurons are often considered the major players in the nervous system being the cell type that converts sensory impulses for interpretation by the brain and return the information to the body so that appropriate actions can be taken. However, these signals would neither be conducted nor interpreted if it were not for the glial cells.

Neurons

There are about 10^{11}-10^{12} neurons in the adult human CNS, a number that seems to vary depending on the quantification technique used since it has been reported that cerebellum alone can contain 10^{11} neurons (Andersen et al., 1992; Williams and Herrup, 1988; Willis Jr, 1993). Neurons differ from most other cells in being excitable, which allows for reception, interpretation, and transmission of signals. Incoming signals are received by the cell body (soma) or the dendrites, which are branchlike protrusions that can account for as much as 90% of the entire neuronal cell surface (Figure 2). Within the neuron, information is conducted by an action potential, which is a rapidly propagated electrical message along the axon. The action potential is a

membrane potential change caused by the flow of ions through ionic channels in the membrane. The action potential, once it reaches the pre-synaptic terminal induces opening of ion channels allowing for influx of Ca^{2+}. Increased levels of intracellular Ca^{2+} triggers the fusion of transmitter vesicles with the pre-synaptic membrane and release of neurotransmitters into the synaptic cleft (Sudhof, 2004). The neurotransmitters bind to and activate receptors on the post-synaptic neuron, which result in a transient change in membrane potential that will act to either increase or inhibit the activity in the post-synaptic cell (Figure 2).

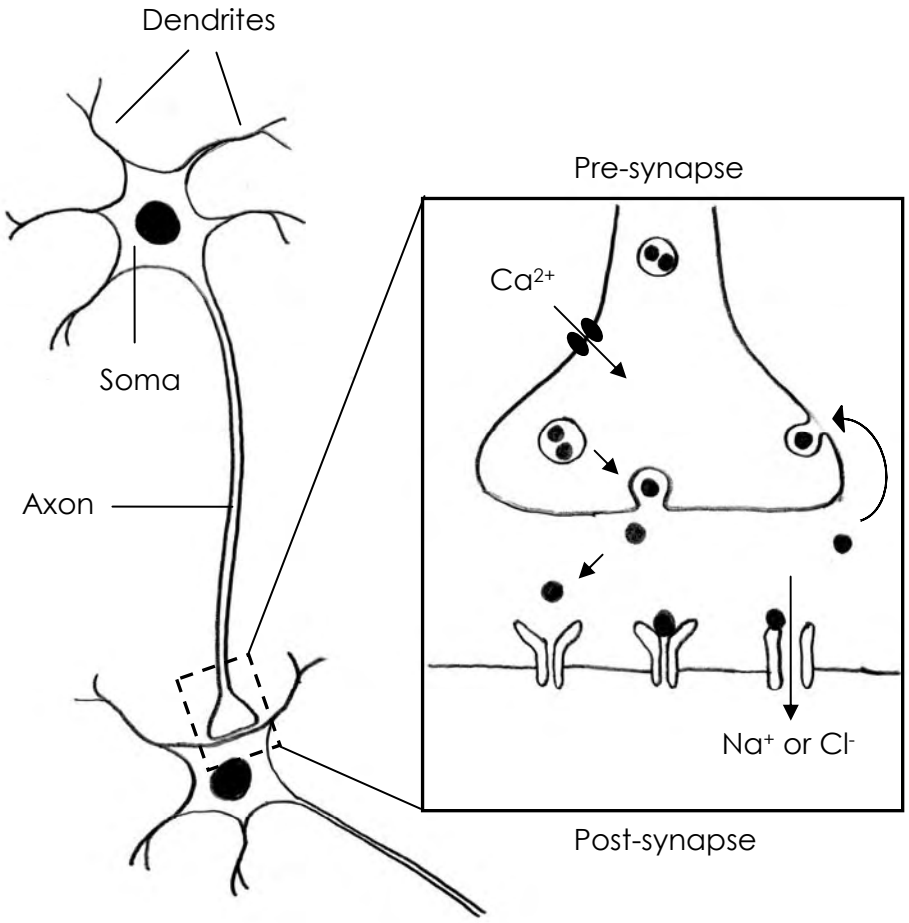

Figure 2: *Neurons consist of a cell body, soma, from which dendrites and an axon protrude. Electric signals are propagated along the axon causing Ca^{2+} influx and transmitter release. The transmitters diffuse over the synaptic cleft and bind to receptors in the post-synapse, which allow for influx of ions and generation of a post-synaptic signal. Excess transmitters can be re-cycled by the pre-synapse.*

Neurotransmitters

Neurotransmitters can be grouped into classical transmitters and neuropeptides. There are nine classical transmitters and with the exceptions of acetylcholine and adenosine triphosphate (ATP), most of them are amino acids [gamma-aminobutyric acid (GABA), glycine, glutamate] or derivates

thereof (dopamine, norepinephrine, epinephrine, serotonin, histamin) (Kutchai, 1993). These classical transmitters are synthesized in their active form by cytoplasmic enzymes. Following release, the membrane potential of the post-synaptic cell needs to be restored by removal or destruction of the neurotransmitters. The neurotransmitter signaling is ended by either enzymatic degradation, diffusion away from the synaptic cleft or more commonly re-cycled by uptake in the pre-synapse (Schwartz, 2000) (Figure 2).

The other class of signaling molecules, the neuropeptides, represent a large family of signaling substances with about 50 members divided into opioids, neurohypophyseal hormones, tachykinins, secretins, insulins, somatostatins, and gastrins (Schwartz, 2000). The neuropeptides are short polymers of amino acids synthesized and packaged in the cell body and transported to the nerve terminal via axonal transport. These substances are synthesized as larger pro-peptides, or even pre-pro-peptides, which can contain several neuropeptides that are activated by cleavage. Neuropeptides usually coexist with one or several classical transmitters complementing or modulating their actions (Lundberg, 1996). As opposed to the classical transmitters, neuropeptide activity is terminated by proteolysis or diffusion. Neuropeptides can be involved in regulation of food intake and body weight [cholecystokinin (CCK), neuropeptide Y (NPY), galanin, agouti-gene related protein (AgRP), cocaine- and amphetamine-regulated transcript (CART)] as well as pain perception (substance P, dynorphin) (Caudle and Mannes, 2000; Dionne et al., 1998; Kalra, 1997; Schwartz et al., 2000). Many neuropeptides are also up-regulated during stress mechanisms and neuronal injury [galanin, NPY, (vasoactive intestinal polypeptide) VIP, calcitonin gene-related peptide (CGRP)] (Hökfelt et al., 2000; Moran and Graeber, 2004).

Macroglia

It has commonly been assumed that glial cells in human brain outnumber the neurons by an order of magnitude (Kandel, 2000; Willis Jr, 1993). However, recent studies in rat show that only 60% of the cells are glial cells (Herculano-Houzel and Lent, 2005). There are three major types of glia in the CNS, astrocytes and oligodendrocytes, here referred to as macroglia, and the microglia (MG), all with unique characteristics. There is also a transient population of glial cells during development called radial glia that are important in directing neuronal migration (Rakic, 1995). Radial glia may also act as precursors for both astrocytes and neurons (Alvarez-Buylla et al., 2001; Parnavelas and Nadarajah, 2001).

Astrocytes

Astrocytes, or star cells, are the most numerous glial cell in the CNS constituting about 40-50% of all glia (Aldskogius and Kozlova, 1998). As

astrocytes mature, they make contact with the brain capillaries via perivascular endfeet (Abbott, 2002) (Figure 3). These structures constitute an important component of the blood-brain-barrier (BBB) and allow for nutrient and metabolite transfer between the neurons and the blood. Astrocytes also maintain the overall homeostasis in the brain by regulating the pH levels, ionic balances, and removal of excess transmitters (Kirchhoff et al., 2001). Astrocytes are often found in close vicinity to synapses where they modulate activity at the synapse by both releasing their own transmitters and by activation via expressed neurotransmitter receptors (Newman, 2003; Ventura and Harris, 1999). Astrocytes connect to each other by gap junctions, pores in the cell membrane that allow ion mediated signaling between the cells (Giaume and McCarthy, 1996). In response to injury, astrocytes become reactive and can undergo hypertrophy and astrogliosis with upregulation of the astrocytic marker glial fibrillary acidic protein (GFAP) and glial scar formation as result. This reactivation of astrocytes is thought to stabilize regions of neuronal injury limiting the damage and thereby protecting the surrounding tissue from damage (Eddleston and Mucke, 1993; Faulkner et al., 2004).

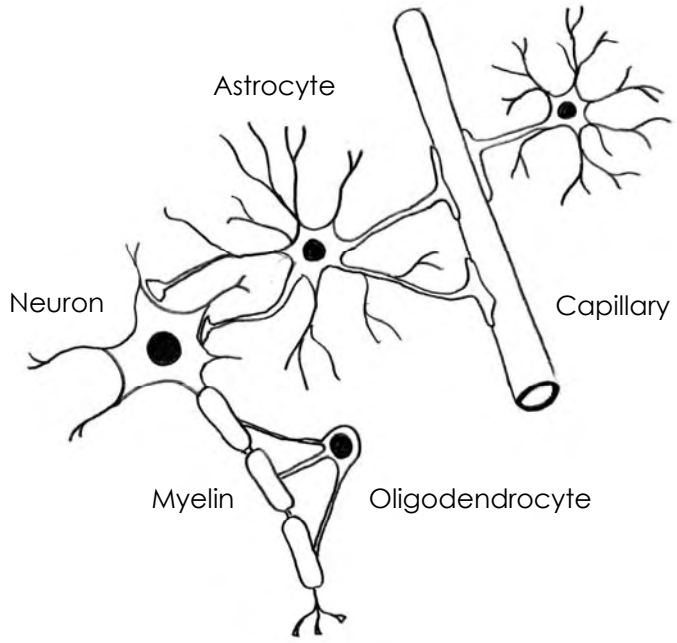

Figure 3: *Neurons and macroglia in the CNS.*

Oligodendrocytes

The main function of the oligodendrocytes is to insulate the axons with myelin allowing for rapid transmission of the action potentials (Ludwin, 1997) (Figure 3). The axon and process-rich white matter of the brain is therefore rich in oligodendrocytes whereas they are less numerous in the gray matter, where most of the neuronal cell bodies are located. It has been shown that

oligodendrocytes and myelin inhibit the regeneration of neurons and axons, most likely mediated by myelin oligodendrocyte glycoprotein (MOG), a myelin specific protein present in the CNS (Ludwin, 1997; Rubin et al., 1994). Inhibition of regeneration is believed to ensure the maintenance of established neuronal circuits, but may hinder regeneration after injury or degeneration (Ludwin, 1997). In multiple sclerosis (MS), the myelin is degraded causing delay of signal transmission and functional impairment.

Microglia and brain macrophages

Microglia are often referred to as brain macrophages due to their resemblance to blood-derived macrophages, both in function and expressed markers (Flaris et al., 1993; Ulvestad et al., 1994). However, "brain macrophages" is broad and with the exception of including the parenchymal MG it also encompasses macrophages that infiltrate the brain, pericytes, and perivascular cells (Guillemin and Brew, 2004). Meanwhile the common denominators for these cells are their immunoregulatory function and mesodermal/monocytic origin (Jordan and Thomas, 1988; Thomas, 1999). However, contradictory theories have been presented regarding the origin of MG, some suggesting a neuroectodermal progenitor (Fedoroff et al., 1997; Hao et al., 1991). In culture, MG has been ascribed the ability to generate neurons, astrocytes, and oligodendrocytes, cells normally derived form the neuroectoderm (Yokoyama et al., 2004). The major differences between the various types of brain macrophages are their spatial location and rate of turn-over. Pericytes are found within the basal lamina of the endothelium in capillaries, not only in the CNS, whereas perivascular cells are located between the vascular endothelium and the neural parenchyma where they comprise a part of the BBB (Thomas, 1999; Williams et al., 2001). The perivascular cells in particular, are continuously replaced with monocytes from the circulation (Lassmann et al., 1993; Rezaie and Male, 1999; Williams et al., 2001). MG on the other hand, migrate into the brain parenchyma during embryogenesis where they represent a stable population with a low rate of turn-over (Lassmann et al., 1993).

During the embryonic phase, microglial cells are large, amoeboid in shape, and take an active part in the formation of neuronal circuits by phagocytosing apoptotic cells and by releasing neurotrophic factors promoting neuronal differentiation and survival (Ashwell, 1990; Ling et al., 1980; Nakajima et al., 2002). When the brain matures, MG enter a resting state characterized by smaller cell bodies, ramification, and withdrawn phagocytic abilities. Although present in all brain regions, the microglial quantity varies between 0.5 and 16% of all cells (Lawson et al., 1990; Mittelbronn et al., 2001). The resting morphology also differs between regions, the bipolar MG being more numerous in the white matter whereas the

stellate form is more common in the gray matter. As the brain ages, the number of activated MG increases (Ogura et al., 1994; Sloane et al., 1999). Quantification of activated MG in young *vs.* old monkeys show significant increase, up to 50%, in some regions (Sloane et al., 1999). The progressive activation is either initiated as a natural process in the aging of MG themselves, or induced by the increasing neuronal loss observed during normal aging (Coleman and Flood, 1987; Streit, 2002).

Microglial activation

If the brain becomes injured or affected by disease, MG are one of the first cells to respond (Streit et al., 1999). During such activation, MG proliferate, retract their processes, increase their cell size and, if required turn into phagocytosing cells (Figure 4). Activated MG up-regulate the expression of a number of immunological markers such as major histocompatibility complex (MHC) class I and II, complement receptor-3 (CR3/CD11b), and Fc-receptors (FcR) (Graeber et al., 1988; Hayes et al., 1987; Streit et al., 1989). Activated MG also increase their production of pro-inflammatory factors i.e. tumor necrosis factor (TNF)-a (Sawada et al., 1989), interleukin (IL)-1β (Giulian et al., 1986), IL-6 (Nakamura et al., 1999), and macrophage inflammatory protein (MIP)-1a (Hayashi et al., 1995) as well as reactive oxygen species (ROS) (Tanaka et al., 1994), and nitric oxide (NO) (Chao et al., 1992). Even though low levels of TNF-a and IL-1 are necessary for normal CNS development (Merrill, 1992), excessive amounts of these cytokines and other MG-derived factors can be detrimental for adjacent neurons. To regulate the steady state number of MG after a pathological stimulus, MG either return to the resting state by down-regulating MHC class II and CR3 or the population is diminished by apoptosis (Gehrmann and Banati, 1995).

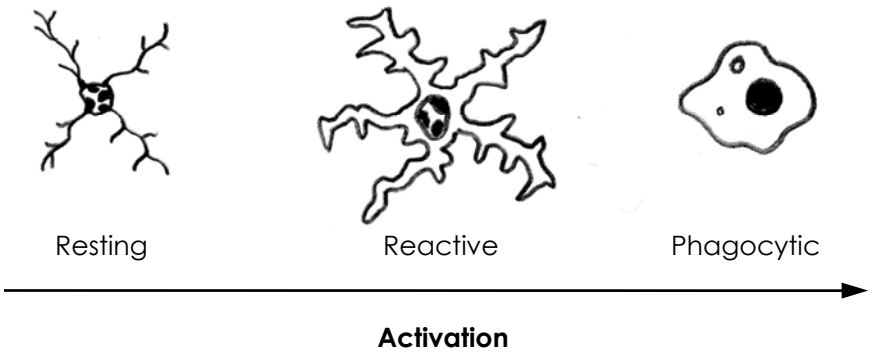

Figure 4: *Resting MG retain a ramified morphology whereas activated MG change their expression pattern and become hypertrophic. If necessary, reactive MG transform into phagocytosing cells (modified from Streit, 1999).*

One of the most common inducers of experimental monocyte and MG activation is lipopolysaccharide (LPS), a component of the outer membrane

of Gram-negative bacteria (He et al., 2002). In the case of gram-negative meningitis, often caused by *Neisseria meningitidis* or *Haemophilus influenzae*, the CNS can encounter LPS (Rock et al., 2004). LPS forms a complex with the serum-derived LPS-binding protein (LBP), which interacts with CD14 and the Toll-like receptor 4 (TLR4) on MG/monocytes that activates intracellular signaling pathways leading to alterations in gene transcription (Guha and Mackman, 2001; Pyo et al., 1998). In cultured MG, LPS induces the expression of outward K^+ channels that are important in the process of MG activation, in particular in the release of NO (Pyo et al., 1997). LPS activation also induces expression and release of pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6 (Nakamura et al., 1999).

Interferon (IFN)-γ is a pro-inflammatory cytokine with antiviral activity, antiproliferative and immunomodulatory effects (Farrar and Schreiber, 1993). In MG, IFN-γ primarily induces expression of MHC antigens and other proteins related to antigen presentation (Frei et al., 1987; Moran et al., 2004; Suzumura et al., 1987). Normally, IFN-γ is only present in the diseased or injured brain as a consequence of BBB disruption and T-cell infiltration. However, neurons can express an IFN-γ like protein (N-IFN-γ) that also up-regulates MHC class I and II expression in macrophages (Ljungdahl et al., 1989; Olsson et al., 1994). In addition, LPS as well as IFN-γ itself has shown to induce IFN-γ expression in macrophages (Di Marzio et al., 1994; Fultz et al., 1993). In combination with LPS, IFN-γ modulates the microglial expression of pro-inflammatory cytokines and chemokines (Häusler et al., 2002; Loughlin and Woodroofe, 1996; Tamai et al., 2003). Microinjection of LPS+IFN-γ into rat hippocampus has shown to induce neuronal apoptosis via inducible nitric oxide synthase (iNOS) and excessive microglial production of NO (Matsuoka et al., 1999).

NEUROGLIAL INTERACTIONS

In the CNS, an active communication between neurons and glia occur, both reliable on soluble factors and direct cell-cell contact. Glial activity is largely determined by the condition and activity of adjacent neurons. In this thesis, I have focused on neuronal interactions with MG rather than macroglia. Therefore, mediators and consequences of neuron-MG interactions will be discussed in more detail below.

Interactions between neurons and astrocytes are largely mediated by neuronal transmitters and the initiation of intracellular Ca^{2+} waves that propagate between astrocytes via gap junctions. Gap junctions are small pores that interconnect the membrane of adjacent cells allowing for direct transmission of ions that can induce release of chemical transmitters, including ATP and glutamate, from astrocytes (Guthrie et al., 1999; Pasti et al., 1997). By up-regulating gap-junctional proteins, astrocytes quickly respond to

the changed homeostasis and activate MG (Aldskogius and Kozlova, 1998; Rohlmann et al., 1993). In addition, ATP released from neurons or astrocytes can promote both astrocytes and MG to produce neurotrophic factors such as basic fibroblast growth factor (bFGF), NGF, neurotrophin (NT)-3, CNTF, and S100β (Rathbone et al., 1999).

As described in the Introduction, oligodendroglial interactions with neurons in the form of myelination of axons are important for increased signal transmission. Oligodendrocytes also provide neurons with trophic support by secretion of factors such as NGF, brain derived growth factor (BDNF), and NT-3 that may act beneficial on neurite maintenance (Byravan et al., 1994; Du and Dreyfus, 2002). Neurons in turn, may regulate oligodendroglial cell differentiation and myelin formation (Kettenmann et al., 1991).

Microglia and neurons

MG can both promote neuronal survival and be detrimental to neurons. The microglial activation rate is highly dependent on environmental factors and communication with other glial cells as well as infiltrating immune cells such as T-cells. The intricate balance between the extremes of the microglial features is also regulated by reciprocal interactions between MG and neurons (Polazzi and Contestabile, 2002). It has been proposed that *in vivo*, the true nature of MG is shifted towards being beneficial for neurons both in the normal brain and after encounter with milder neuronal insults (Streit, 2002). However, MG-mediated neurotoxicity may dominate when signals from the surrounding neurons overwhelmingly indicate irreversible cell damage (Streit, 2000). To mediate neuroprotection, MG secrete neurotrophins (NGF, BDNF, NT-4/5) (Elkabes et al., 1996), hepatocyte growth factor (HGF) (Hamanoue et al., 1996), cytokines (IL-3, IL-6) as well as growth factors [transforming growth factor (TGF)-β, bFGF] (Kreutzberg, 1996; Nakajima and Kohsaka, 2001; Nakajima et al., 2002; Shimojo et al., 1991) where the expression of some of these are upregulated at MG activation (Kreutzberg, 1996).

Neurons and MG interact via the chemokine fractalkine and its receptor CX3CR-1, expressed by MG (Harrison et al., 1998). In the brain, fractalkine is expressed primarily by neurons either membrane bound or released as a soluble factor (Harrison et al., 1998). Although it has been reported that MG and astrocytes also express fractalkine in a normal state or when stimulated with specific pro-inflammatory mediators (Yoshida et al., 2001; Zujovic et al., 2000). When neurons are damaged they release soluble fractalkine, which acts as a chemoattractant on macrophages, T-cells, and NK-cells, presumably also on MG suggesting a role in recruitment of immunomodulatory cells (Mizuno et al., 2003). On encounter, the highly

adhesive membrane bound form of fractalkine induces increased mobilization of intracellular Ca^{2+} in MG that reduce the expression of pro-inflammatory mediators favoring neuronal survival (Harrison et al., 1998; Mizuno et al., 2003).

Injured neurons release transmitters including ATP and glutamate, which activates MG through purinergic receptors and glutamate receptors respectively (Ferrari et al., 1996; Haas et al., 1996; Noda et al., 2000; Walz et al., 1993). The microglial response to these signals leads to upregulation of the expression of immediate early genes and subsequent release of cytokines such as TNF-a (Hide et al., 2000; Priller et al., 1995). TNF-a in turn induce an inflammatory response that may result in ensuing apoptosis. However, the microglial release of TNF-a and other cytokines can be downregulated by VIP, which is released by depolarized neurons and induces release of neurotrophic factors such as activity-dependent neurotrophic factor (ADNF) and NT-3 (Kim et al., 2000). ATP also initiates an outward directed K^+ conductance in MG, which is important for the initiation of the activation process (Walz et al., 1993). In peripheral MG, ATP-induced activation has shown to play an important role in mediating pain following nerve damage (Tsuda et al., 2003). MG can also respond to K^+ released by injured neurons by upregulation of MHC class II expression (Gehrmann et al., 1993).

Platelet-activating factor (PAF), synthesized by neurons, is also an important messenger in neuron-MG interactions. The signal transmission occurs via PAF receptors expressed on MG (Aihara et al., 2000; Mori et al., 1996). Activation of these receptors cause an increase in intracellular Ca^{2+} and arachidonic acid release by MG (Mori et al., 1996). Arachidonic acid is a lipid messenger that, together with its metabolites (prostaglandins, prostacyclin, and thromboxanes), modulates inflammatory responses.

The removal of cellular debris and apoptotic cells after a trauma can in a sense be seen as a neuroprotective act allowing for remyelination and neuronal regeneration (Friede and Bruck, 1993; Prewitt et al., 1997). Macrophages are triggered to recognize and phagocytose apoptotic cells at an early stage by recognition of phosphatidylserine (PS) that translocates from the inner leaflet of the cell membrane to the outer early in the apoptotic process (Fadok et al., 1992). The recognition of PS has shown to reduce the synthesis of NO and TNF-a by LPS-activated MG in culture (De Simone et al., 2003) and to increase the levels of prostaglandin E_2 (PGE_2) that may act suppressive and down-regulate MG activation (Zhang and Rivest, 2001). The phagocytic MG also release anti-inflammatory factors such as TGF-β1 and IL-10, which suppress the pro-inflammatory response (Minghetti et al., 1998).

Many studies have been performed regarding consequences of MG activation, but not much is known about the mechanisms involved in mediating a down-regulated phenotype of MG in the intact brain. It has been presented that the immunoglobulin superfamily member CD200 (OX2) expressed in neurons interacts with CD200R expressed on MG (Hoek et al., 2000). Interactions between CD200 and its receptor have shown to keep the MG in a resting state and macrophage/MG activation is greatly enhanced in mice that lack CD200 (Hoek et al., 2000).

Neuron/microglia interactions in neurodegenerative disease

Activated MG and MG-derived pro-inflammatory factors have been implicated as exacerbators of neurogegenerative and autoimmune diseases including Parkinson's disease (PD), Alzheimer's disease (AD), and MS (Benveniste, 1997; McGeer and McGeer, 2004). The causative agents of these diseases are not fully identified although normal aging, genetic mutations, environmental factors, and viruses have been suggested (Gilden, 2005; Hoyer, 2002; Huang et al., 2004). The initial role of inflammation in degenerative diseases has been described as beneficial (Correale and Villa, 2004). However, data from human studies indicate that inflammatory mediators contribute to exacerbation of these diseases. Administration of anti-inflammatory agents has shown to act beneficial on both AD and PD progression in humans (Gao et al., 2003; in t' Veld et al., 2001). The inflammatory reaction in neurodegenerative disorders can be induced by molecules released from, or associated with, injured neurons, protein aggregates or dysregulation of inflammatory control mechanisms (Wyss-Coray and Mucke, 2002). For example, deposition of amyloid β peptides (Aβ), which is a characteristic in the pathology of AD, can activate MG via the receptor for advanced glycation end products (RAGE) or scavenger receptors (El Khoury et al., 1996; Paresce et al., 1996; Yan et al., 1999), which enhances the production of inflammation-mediating cytokines (Yates et al., 2000). Aβ aggregates also activate the complement system, which is proposed to be a beneficial process preventing Aβ-induced neurotoxicity by induction of microglial phagocytosis and elimination of toxic deposits and debris (Wyss-Coray et al., 2002). In PD, there is a clear association between loss of dopaminergic neurons in substantia nigra and MG activation. In animal models of this disease, inhibition of MG activation can halt the neurodegenerative process, which suggests that MG play an essential role in PD progression (Orr et al., 2002).

It seems as if the inflammatory process is aimed at neutralizing the inflammatory trigger. However, if the response persist over extended periods of time it could have detrimental consequences to the surrounding cells, in particular neurons (Hanisch, 2002).

CELL DEATH

Cell death is commonly divided into apoptosis and necrosis. However, the boundary between these distinct forms of death is rather vague as a whole spectrum of death programs and intermediate processes have been suggested (Leist and Nicotera, 1997; Nicotera et al., 1999; Proskuryakov et al., 2003). Necrosis is generally considered a passive and unregulated process primarily involved in pathological situations, although there are reports where necrosis is part of normal events as well (Holler et al., 2000; Mayhew et al., 1999; Murdoch et al., 1999). The morphological hallmarks of necrosis are swelling and rapid disruption of organelles and the plasma membrane (Figure 5). As a result of the plasma membrane disintegration, the cellular contents are released and cause ensuing inflammation in the surrounding tissue (Proskuryakov et al., 2003). On the contrary, apoptotic cells maintain an intact cell membrane throughout the process, which is characterized by morphological features such as cell shrinkage, nuclear condensation, DNA fragmentation, and generation of apoptotic bodies (Figure 5). *In vitro*, apoptosis is often followed by secondary necrosis, which involves lysis of the cellular remains. *In vivo*, the secondary necrosis is effectively prevented by phagocytic engulfment of apoptotic cells. An insult can often initiate either apoptosis or necrosis depending on the intensity of the impairment, suggesting that there are common key factors involved in the respective pathway.

Apoptosis

Apoptotic cell death was first observed in the mid 60's when John Kerr performed histological studies on ischemic liver. Apart from classical necrosis, he discovered a histologically different type of cell death, initially termed shrinkage necrosis after its characteristic morphology (Kerr, 1965). However, as it occurred that this type of cell death was widely involved in both normal and pathological processes, the term apoptosis was mounted (Kerr et al., 1972). Over the years, it has become clear that apoptosis is an active process, which is both genetically regulated and orchestrated by environmental factors. The apoptotic process is necessary for normal development of an organism where it contributes to the massive neuronal death during development, removal of interdigital web tissue, metamorphosis of the tad pole and much more (Wyllie et al., 1980). In the adult, apoptosis is important for maintenance of normal tissue homeostasis. Imbalances in the regulation of apoptosis are the cause of a number of pathological events including cancer and neurodegenerative, cardiovascular or autoimmune diseases. Understanding the mechanisms of apoptosis is necessary in order to define better therapeutic strategies for these diseases.

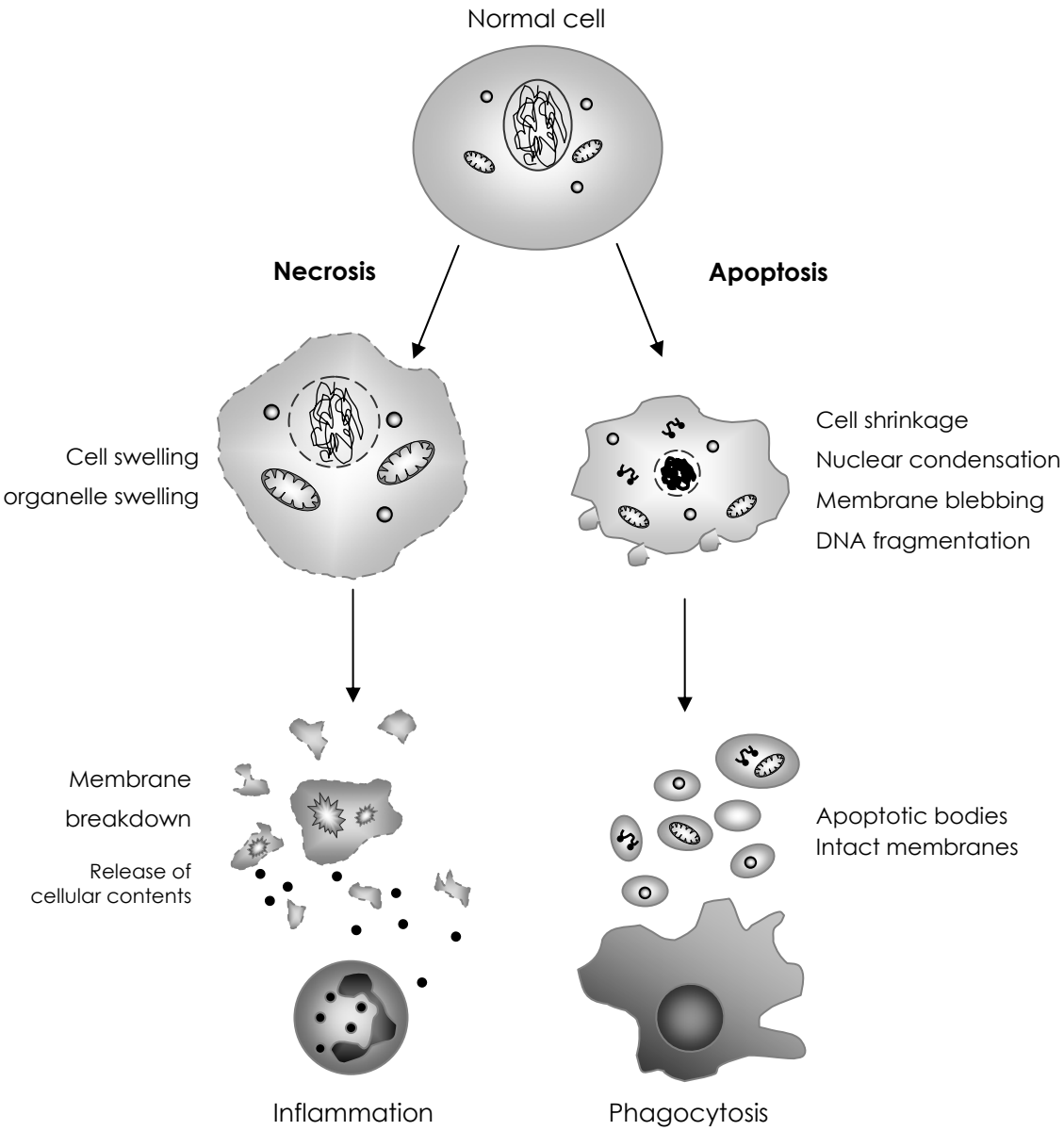

Figure 5: *Morphological characteristics of necrosis and apoptosis.*

Apoptosis signaling

There is an intricate cascade of events that coordinate the ensuing elimination of a dying cell (Zimmermann et al., 2001). Numerous signaling cascades can instruct the cell to initiate apoptosis. However, these signals converge into only two major pathways which mediate apoptosis, these are the death receptor (extrinsic) pathway and the stress-induced mitochondrial (intrinsic) pathway (Ashkenazi and Dixit, 1998; Creagh and Martin, 2003). Some apoptosis inducers preferentially engage a particular signaling pathway, however many stresses, for example UV irradiation, are complex and can induce apoptosis by engaging multiple initiating mechanisms (Norbury and Zhivotovsky, 2004).

Caspases

The central component of the apoptotic machinery is the caspase family of aspartate-specific cysteine proteases (for a comprehensive review of these enzymes see Chang and Yang, 2000). As the name implies, these enzymes harbor a cysteine in their catalytic site and preferentially cleave their substrates after an aspartate residue (Alnemri et al., 1996). Caspases are well conserved proteins found in organisms ranging from *Caenorhabditis (C.) elegans* to humans. To date, 14 mammalian caspase members have been cloned, of which 11 human enzymes are known (Thornberry and Lazebnik, 1998) (Figure 6).

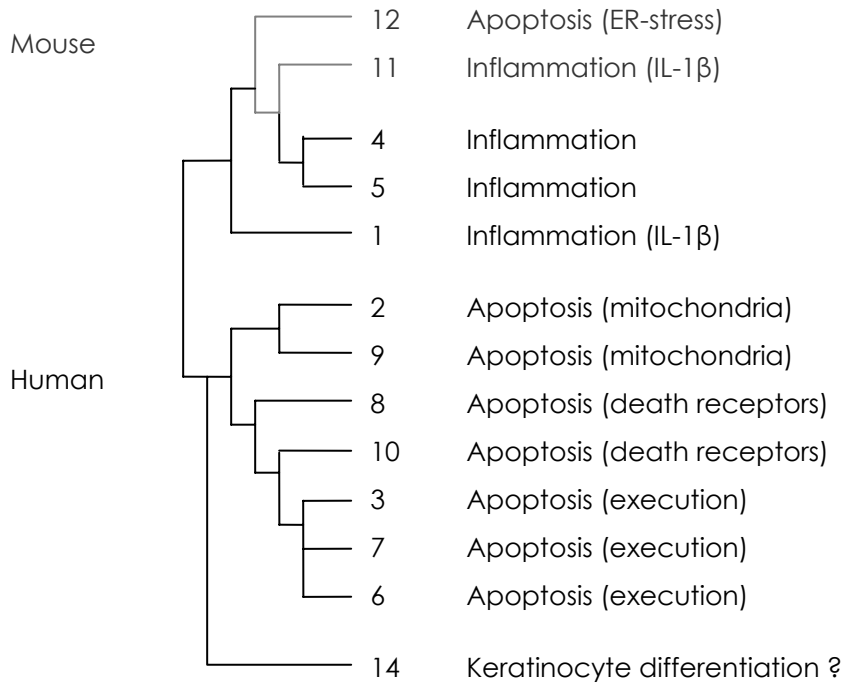

Figure 6: *Phylogenetic relationship among the mammalian caspase-family members. Caspase-13 [found in cattle (Koenig et al., 2001)] has not yet been classified in correlation to the other caspases. (Adapted from www.alexis-e.biz).*

Caspases -1, -4, -5, and -11 are primarily involved in cytokine processing and inflammation whereas caspases -2, -3, -6, -7, -8, -9, and -10 are mediators of apoptosis (Slee et al., 1999; Tschopp et al., 2003). The apoptotic caspases can be further grouped into initiator and executioner caspases depending on their protein structure and where in the apoptosis cascade they exert their action. Like nearly all proteases, caspases are normally present in the cell as inactive pro-enzymes, which require cleavage for activation (Yang et al., 1998). The two caspase classes, initiator and executioner use different modalities to ensure activation. Inactive initiator caspases (caspases -2, -8, -9, and -10) are present as monomers. Upon recruitment to macromolecular activation complexes, the initiator caspases dimerize and by this means transactivate (Boatright et al., 2003). Initiator caspases then proceed to cleave and thereby activate downstream executioner caspase dimers

(caspases -3, -6, and -7) (Figure 7 and 8). The executioner caspases complete the apoptosis progression by disassembly of a number of cellular structures. It has been shown that activated (cleaved) caspase-3 is essential and sufficient to complete apoptosis (Nicholson et al., 1995). In this process, caspase-3 initiates cleavage of chromosomal DNA by activation of the DNA fragmentation factor (DFF) (Liu et al., 1997; Nagata et al., 2003). Caspase-3, and -7, are also involved in cleavage and inactivation of poly(ADP-ribose) polymerase (PARP), a multifunctional nuclear enzyme that is important in DNA repair (Herceg and Wang, 2001; Lazebnik et al., 1994). Caspase-6 contributes to the final stages of apoptosis by initiating the collapse of the nuclear lamina resulting in chromatin condensation (Orth et al., 1996)

The death receptor (extrinsic) pathway

The initiator caspases -8 and -10 are involved in the extrinsic apoptosis pathway typically triggered by binding of extracellular ligands to death receptors such as Fas (CD95/Apo-1) and TNF receptor 1 (TNFR1) (Figure 7) (Nagata and Golstein, 1995; Sheikh and Huang, 2003). However, stresses such as UV irradiation can activate death receptors in a ligand-independent manner (Sheikh et al., 1998).

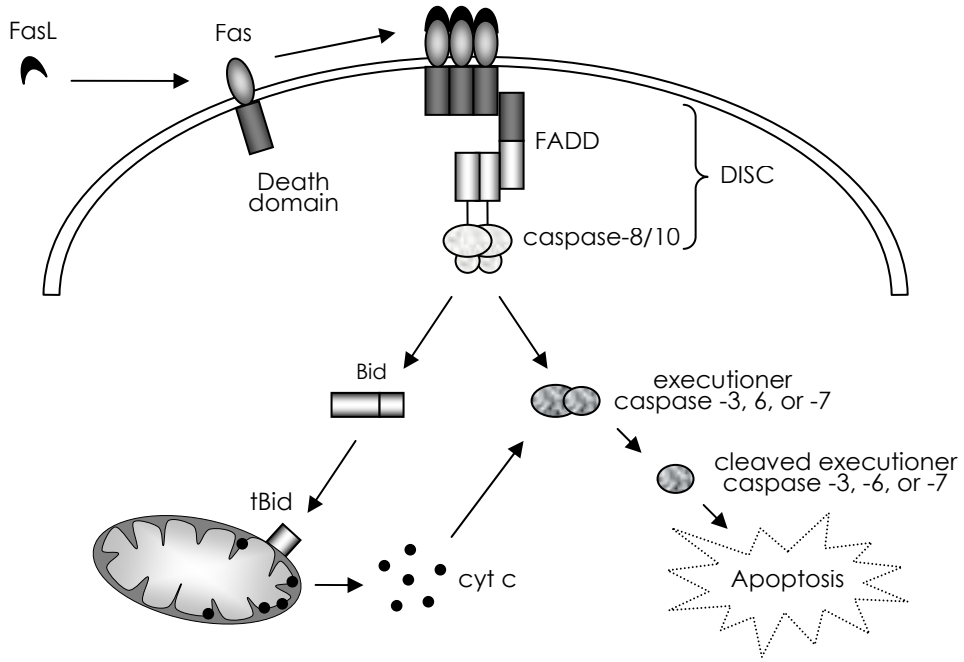

Figure 7: *The extrinsic apoptosis pathway exemplified by binding of FasL to the Fas receptor. Multiple Fas death domains recruit the adapter molecule Fas-associated death-domain protein (FADD) that initiates dimerization and activation of caspase-8 or -10. Caspase-8/10 further activates executioner caspases either by direct cleavage or indirectly through a Bid-mediated activation of the mitochondrial pathway. The executioner caspases dismantle the cell by cleavage of important structural and functional molecules.*

The death receptors belong to the TNF/NGF receptor family that shares a common domain in the cytoplasmic part of the protein called the death domain (Ashkenazi and Dixit, 1998). Binding of ligand generally leads to trimerization of the receptors followed by binding of the cytoplasmic death domains to corresponding structures on receptor specific adapter molecules (Chinnaiyan et al., 1995). The adapters then recruit caspase-8 or -10 to form a complex known as the death-inducing signaling complex (DISC) (Muzio et al., 1998). The DISC initiates caspase-8/10 activation and further engagement of downstream executioner caspases (Figure 7). The extrinsic apoptosis pathway is generally activated in T-cell selection and elimination of virally infected cells.

The mitochondrial (intrinsic) pathway

The mitochondrial pathway is the predominant route for neuronal apoptosis and is the route generally engaged following cellular stresses (Stefanis, 2005). Here, caspase-9, apoptotic protease activating factor-1 (Apaf-1), and cytochrome c (cyt c) form a complex called the apoptosome, which further activates downstream executioner caspases (Li et al., 1997; Zou et al., 1997) (Figure 8). Cyt c is an electron transporting protein normally located to the inner membrane of the mitochondria where it is part of the respiratory chain. It has been proposed that release of cyt c from mitochondria is required for cells to undergo apoptosis (Liu et al., 1996). During a stress response, mitochondria also release other proteins that are important in the ensuing apoptosis process. For example, apoptosis inducing factor (AIF) and endonuclease G are released and translocate to the nucleus where they mediate DNA fragmentation (Penninger and Kroemer, 2003) whereas second mitochondria-derived activator of caspase (Smac) and Omi binds to inhibitor of apoptosis proteins (IAPs) thus preventing their inhibition of caspases (Du et al., 2000; Hegde et al., 2002). Furthermore, the release of mitochondrial proteins to the cytoplasm is also regulated by pro- and anti-apoptotic members of the B-cell leukaemia/lymphoma 2 (Bcl-2) family of proteins (Gross et al., 1999). Modifications such as cleavage, dimerization, and phosphorylation of the Bcl-2 members are important in the regulation of the cell fate following induction of the mitochondrial apoptosis pathway (Ito et al., 1997; Li et al., 1998; Wei et al., 2000).

In some cell types, cross-talk between the extrinsic and the intrinsic pathway occur via the pro-apoptotic Bcl-2 family member BH3-interacting domain death agonist (Bid) that is activated following cleavage by caspase-8 (Li et al., 1998; Luo et al., 1998). Truncated Bid (tBid) then translocates to the mitochondria where it induces cyt c release (Wei et al., 2000) (Figure 7). In addition, the extrinsic and intrinsic apoptosis pathways ultimately converge at the level of caspase-3.

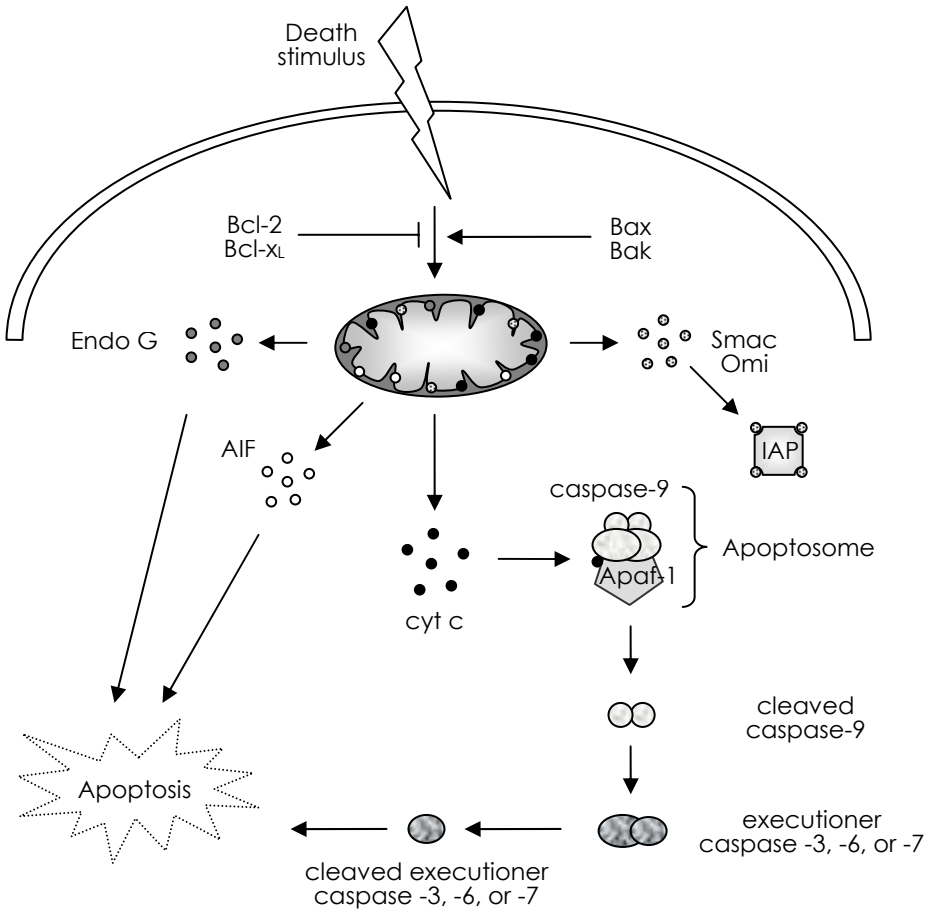

Figure 8: *Stress or cytokine induced apoptosis signals activate the mitochondrial pathway. Members of the Bcl-2 family of proteins regulate the release of mitochondrial proteins such as Endo G, AIF, cyt c, Smac, and Omi. Endo G and AIF induce DNA fragmentation whereas Smac and Omi bind to IAP to prevent it from binding caspases. Cyt c forms a complex, the apoptosome, together with Apaf-1 and caspase-9, which induces activation of caspase-9, cleavage of executioner caspases and ensuing apoptosis.*

Stress-induced apoptosis

Apoptosis induced by external stimuli such as UV irradiation, pro-inflammatory cytokines, and certain mitogens induce apoptosis by engagement of the mitogen-activated protein kinases (MAPKs). MAPKs are serine/threonine kinases that phosphorylate substrates such as transcription factors and other proteins involved in apoptosis regulation (Deng et al., 2001; Fuchs et al., 1998; Ip and Davis, 1998). Different types of MAPKs can be activated in response to specific stimuli. For example, the extracellular signal-regulated kinase (ERK) MAPKs are preferentially activated by growth factors and neurotrophic factors, while the p38 kinases and the c-Jun N-terminal protein kinases (JNKs) are rather activated by stress-inducing factors such as UV irradiation or inflammatory cytokines (Kulms and Schwarz, 2002; Sluss et al., 1994). In reference to the apoptotic pathways studied in this thesis, I will focus this part

of the introduction on the JNK MAPKs, also referred to as stress-activated protein kinases (SAPKs). Generally, the initiating stressors that activate the JNK pathway bind to receptors that recruit adaptor molecules such as members of small GTPases of the Rho-family (Rac, Rho, cdc42) (Coso et al., 1995; Minden et al., 1995). These events further activate a cascade of kinases, including MAP/ERK kinase kinase 1-4 (MEKK1-4) and MAPK kinase 4 and 7 (MKK4/7) that phosphorylate the three JNKs at residues Thr183 and Tyr185 (Lin et al., 1995; Tournier et al., 1997) (Figure 9).

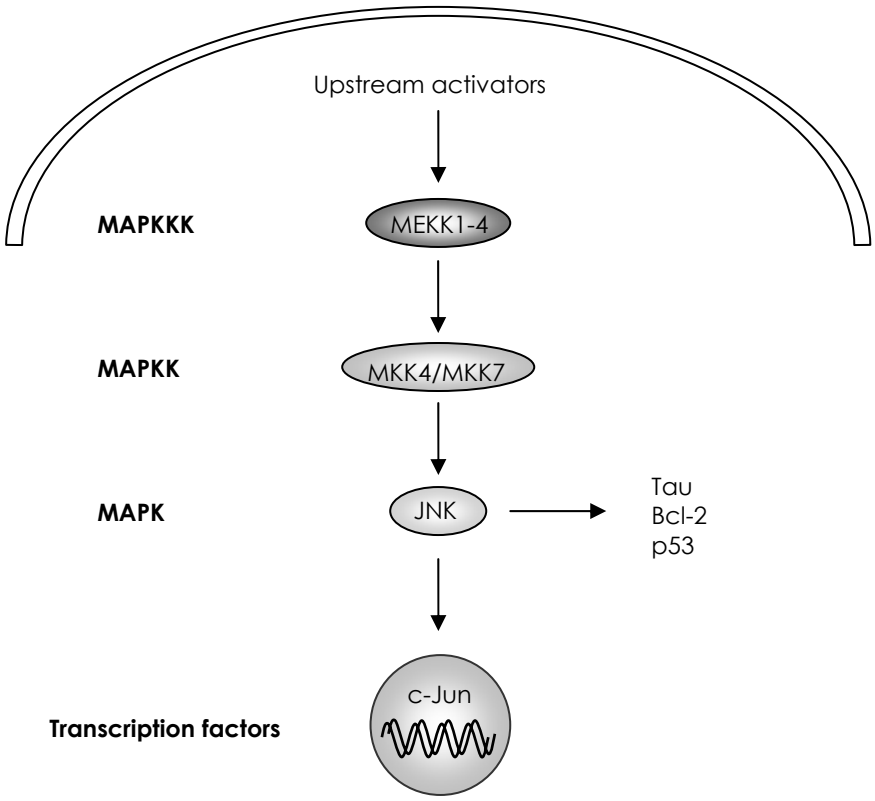

Figure 9: *Protein kinase pathways activating the JNK/SAPK MAPKs that further phosphorylates downstream effectors such as the c-Jun transcription factor, the Tau filament protein or the anti-apoptotic regulator Bcl-2.*

Genetic modification of the *Jnk3* gene in mice indicates that JNKs, and JNK3 in particular, are required for induction of neuronal apoptosis (Yang et al., 1997). Other studies propose that JNK activation facilitates, but does not induce apoptosis (Lin, 2003). The JNK protein kinases are encoded by three genes, *Jnk1*, *Jnk2*, and *Jnk3*, where *Jnk3* is uniquely expressed in neurons (Gupta et al., 1996). The genes are alternatively spliced to generate ten isoforms, which are active as dimers that either phosphorylate cytoplasmic substrates or translocate to the nucleus where they regulate transcription via phosphorylation of c-Jun, ATF-2, and other transcription factors (Gupta et al., 1996; Maundrell et al., 1997; Reynolds et al., 1997). JNK has long been considered the only kinase reported to phosphorylate c-Jun at residues Ser63

and Ser73, important in regulating the transcriptional activity of c-Jun and to mediate apoptosis (Behrens et al., 1999; Minden et al., 1994). However, it has been suggested that c-Jun may be phosphorylated at these sites by a cyclin-dependent kinase (CDK)-like kinase (Besirli and Johnson, 2003). c-Jun regulates transcription of both pro- and anti-apoptotic gene products and depending on the sum of the signals, the cell will either undergo apoptosis or survive (Shaulian and Karin, 2002).

AIMS OF THE THESIS

The overall aim of this thesis was to study the interplay between cells in the CNS with focus on microglia-neuronal interactions. In particular, the balance between the neurotoxic and neuroprotective features of MG was of interest. In the course of these studies, the following themes emerged:

Establishment and characterization of a complex cell culture model, suitable for *in vitro* studies of interactions between CNS cells in a milieu resembling the *in vivo* situation.

Investigation of the neurotoxic and neuroprotective roles of MG/monocytes by developing a model for neuronal injury and by introducing activated THP-1 monocytes to SH-SY5Y neuroblastoma cells in co-culture. In this process, the ImageXpress imaging and analysis system was evaluated as a means to morphologically detect and quantify apoptotic neurons.

METHODOLOGICAL CONSIDERATIONS

Detailed reports on the materials and methods used in this thesis are provided in the original articles. Here, I give a more general description and discussion of the methods.

CELL CULTURING

Primary cells

When working with cell culture systems, it is often desirable to mimic the *in vivo* situation as much as possible. Using primary cells, i.e. cells derived directly from the tissue of interest is a common way of simplifying a system and enhancing the experimental control. The complexity can be increased by co-culturing different cell types, either in different compartments restricting the signaling to soluble factors or in direct contact with each other allowing for contact-mediated communication. Co-cultures can be maintained either as monolayer cultures or as three dimensional aggregates as described below.

Aggregate cultures

In paper I, fetal brain cell aggregates were generated from cortex and hippocampus dissected from mice at embryonic day (ED) 16-17. The tissue was dissociated mechanically instead of enzymatically since we observed that trypsin generated a heterogenous population of dead and viable cells whereas centrifugation of mechanically dissociated tissue resulted in a larger proportion of viable cells. The mixed brain cells were resuspended in Neurobasal™ medium (Brewer, 1995; Brewer et al., 1993) complemented with B27 supplement (Svendsen et al., 1995), a combination that promoted growth of primary neurons and resulted in stable aggregates, both in size and cell contents. Replacing B27 with the glia promoting G5 supplement favored glial cells and gave larger aggregates that contained a larger proportion of macroglia. The cells were seeded in a volume of 4 ml in Ehrlenmyer flasks (25 ml) that were placed on a gyratory shaker to initiate the aggregate formation (Figure 10). As reported previously (Honegger, 1985; Moscona, 1961), the agitation speed is crucial for proper aggregate formation, therefore the initial speed of 68 rpm was increased daily by 2-3 rpm until reaching a final speed of 81 rpm. The aggregate formation was initiated within a few hours and after 4 days, the cultures that now contained small and irregularly shaped aggregates were transferred to 50 ml flasks and

expanded to 8 ml. Fresh nutrients were applied by medium replenishment 2-3 times a week. Aggregates were kept in culture for more than 70 days although the culture period chosen for characterization in Paper I ranged from day 4 to 56.

To investigate whether the aggregates contained uncommitted neural precursor cells, cultures were supplemented with epidermal growth factor (EGF). EGF is a mitogen that is frequently used for proliferation of precursor cells (Reynolds and Weiss, 1992; Svendsen et al., 1995; Wong and Guillaud, 2004). Another mitogen that is potent for neurons and glial cells is bFGF (Vescovi et al., 1993). When stable aggregates had formed (> day 14), 20 ng/ml EGF was added to the cultures. EGF-treatment increased the aggregate size by cell proliferation and growth of an outer layer surrounding the original aggregate.

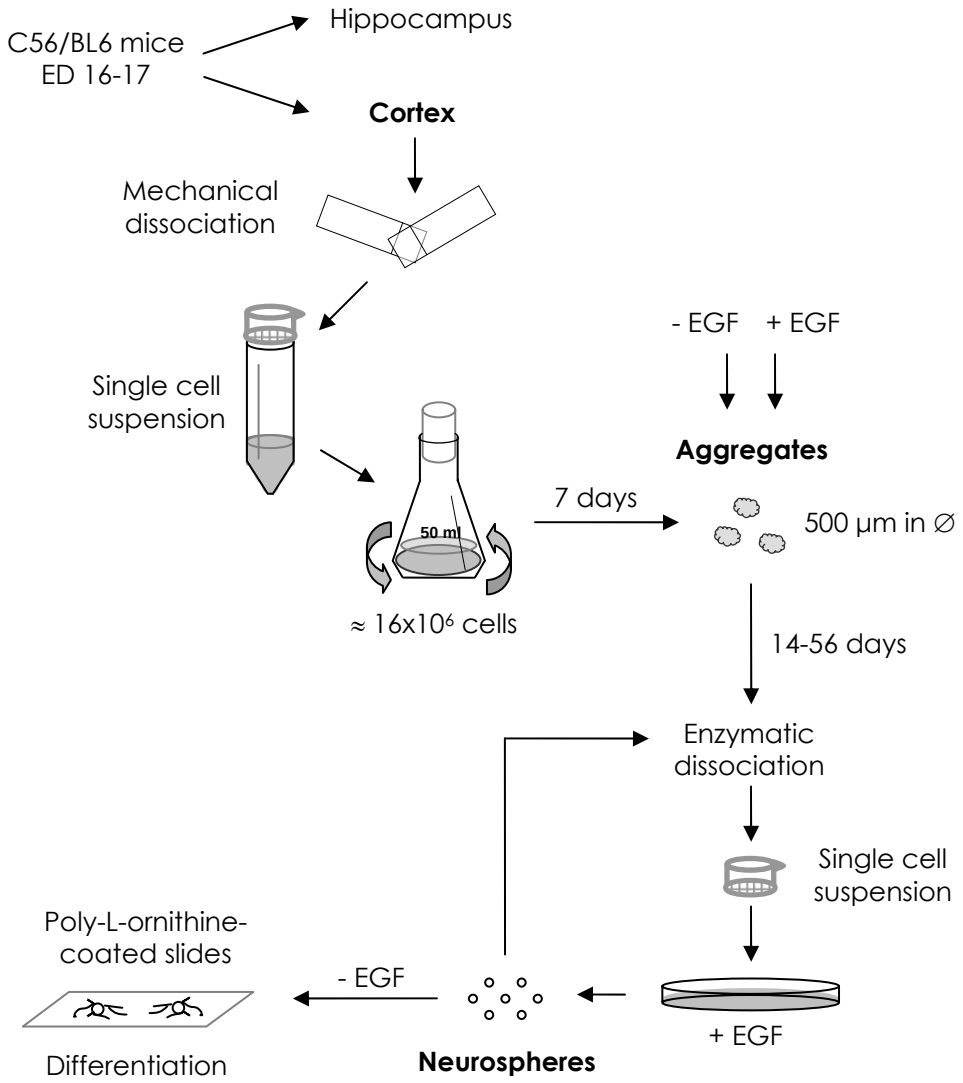

Figure 10: *Generation of fetal brain cell aggregates and neurospheres.*

Neurospheres

To investigate the EGF-induced proliferating cells, and to possibly identify precursor cells harboured in the aggregates, untreated or EGF-treated aggregates were enzymatically dissociated in a Trypsin-EDTA solution. The cells were seeded in Petri dishes as single cells cultured in Neurobasal-B27 supplemented with EGF. The cells proliferated into cell clusters, or neurospheres, which could be further dissociated and re-seeded as single cells generating clonally expanded neurospheres (Figure 10). When allowed to adhere to poly-L-ornithine-coated cover slips in the absence of EGF, the neurospheres differentiated. After fixation, the differentiated cells were immunocytochemically identified as neurons, astrocytes, and oligodendrocytes.

Cell lines

Even though immortalized cells may be less representative of *in vivo* conditions than primary cultures there are some major advantages with cell lines. The use of cell lines do not require experimental animals, they are pure and easy to maintain in culture for extended periods of time due to their high stability. Many mechanistic questions can be answered using cell lines as an initial model system. After strengthening a hypothesis, further studies can be performed using primary cells, complex cultures or *in vivo* models.

SH-SY5Y cells

In Papers II-IV, the human neuroblastoma cell line SH-SY5Y was used in its undifferentiated stage. The SH-SY5Y cell line is originally subcloned from the neuronal cell line SK-N-SH, which is derived from a metastatic human neuroblastoma (Biedler et al., 1973; Biedler et al., 1978). SH-SY5Y cells are widely used as experimental models for neuronal apoptosis, differentiation, and neurogenesis (Neumar et al., 2003; Simpson et al., 2001).

THP-1 cells

In Paper III, human THP-1 monocytes originating from an acute monocytic leukemia were used as a model for monocytes/macrophages (Tsuchiya et al., 1980). There are reports describing the expression pattern of THP-1 cells in relation to primary monocytes, which can be compared to gene expression profiles on MG (Duke et al., 2004; Kohro et al., 2004). A difference between primary monocytes and THP-1 cells is that the former needs stimulation with M-CSF and GM-CSF to survive *in vitro* whereas THP-1 cells divide indefinitely without such stimulation (Kohro et al., 2004). THP-1 cells were used either unstimulated or activated with 0.5 µg/ml LPS, 1.25 ng/ml IFN-γ or a combination of both for 24 hours (h). Upon activation, THP-1 cells become adhesive and express a plethora of pro-inflammatory mediators including IL-1β, IL-6, and TNF-α (Klegeris and McGeer, 2001).

SH-SY5Y/THP-1 co-cultures

In Paper III, SH-SY5Y cells were either cultured in sterile filtered (0.22 µm) conditioned media (CM) from unstimulated/stimulated THP-1 cells or co-cultured in direct contact with THP-1 cells for 24, 48, and 72h. To avoid confluency, the cells were plated at different densities depending on the culture period. However, the SH-SY5Y:THP-1 ratio at seeding was equal (2:1) for all three co-culture time points.

CELL DEATH

Induction of neuronal injury

There are several ways of inducing neuronal cell death experimentally depending of the rate of injury requested and the hypothesis model. Over-activation of neurons with excitatory neurotransmittors, such as glutamic acid (glutamate), N-methyl-D-aspartate (NMDA), kainic acid (KA) or a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) induce neuronal toxicity via glutamate receptors. Neuronal cell death can also be induced by limiting the nutrient supply by removal of serum or by blocking the ATP synthesis by impairment of the respiratory chain.

Rotenone

In Paper II, neuronal apoptosis was induced by incubating the SH-SY5Y cells with the mitochondrial toxin rotenone at different concentrations (0.1-3 µM) for 24h. Rotenone is a naturally derived pesticide that induces apoptosis by inhibiting complex I in the respiratory chain of mitochondria. This has two detrimental consequences 1) accumulation of electrons in the lumen with subsequent formation of free radicals and 2) compromised ATP synthesis. Rotenone is often used to study degeneration of dopaminergic neurons as it has been shown that rotenone evokes PD-like symptoms when administered to rats (Alam and Schmidt, 2002; Betarbet et al., 2000). Another complex I inhibitor is 1-methyl-4-phenylpyridinium (MPP^+), the neurotoxic metabolite from 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), which specifically act on dopaminergic neurons by using the dopamine transporter to enter the cells. We chose to use rotenone as it is degraded after 24h, whereas MPTP/MPP^+ accumulates in the cells and hence require more extensive precautions in the lab.

UV irradiation

In Paper IV, neuronal damage was induced by UV exposure of SH-SY5Y cells (Mielke and Herdegen, 2000). The advantage with this injury model is that no change of media is necessary between the induction and the harvest, therefore factors present in the media will be unchanged. The insult was correlated to early [phosphorylated JNK (p-JNK), phosphorylated c-Jun (p-c-Jun), cyt c-release] and late (activated caspase-3, nucleosome formation)

death markers after incubations ranging from 5 minutes to 48h after treatment with different doses of UV radiation (Figure 11).

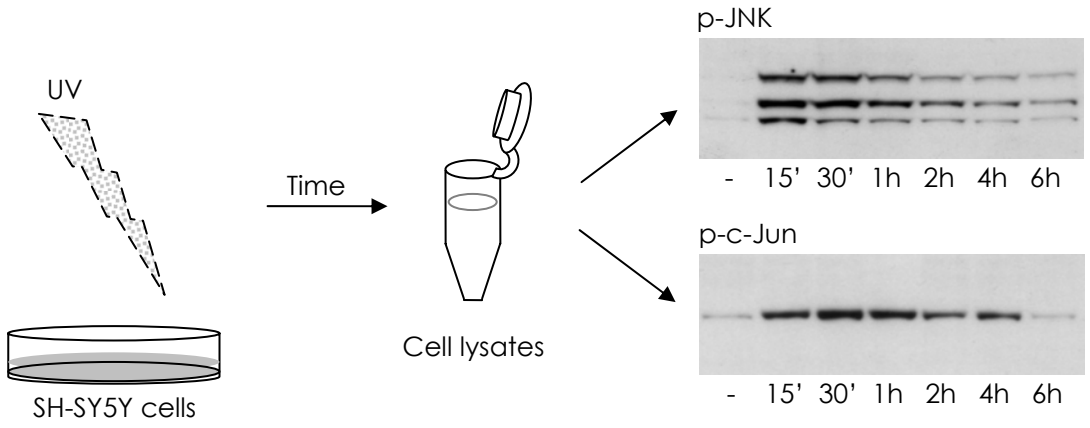

Figure 11: *UV irradiation of SH-SY5Y neuroblastoma cells and subsequent Western blot detection of proteins in the cell lysates.*

To analyze the down-stream effects of JNK-inhibition, 10-30 μM of the JNK inhibitor SP600125 (Bennett et al., 2001) was added to the SH-SY5Y cells 1h prior to UV treatment. The same procedure was followed for the CDK-inhibitor roscovitine (Meijer et al., 1997).

Cell death assays

Many widely used methods for determination of cell death are based on the uptake or exclusion of vital dyes like Trypan blue, propidium iodide or ethidium bromide where dead and viable cells are discriminated by differential staining (Cook and Mitchell, 1989; Jones and Senft, 1985; Yuhas et al., 1974). However, these approaches are not suitable for large numbers of samples and they do not account for dead cells that may have lysed, which may lead to underestimation of the rate of cell death.

LDH release

In Papers III and IV, necrosis was detected by measuring the amount of lactate dehydrogenase (LDH) released to the culture medium. LDH is a cytoplasmic enzyme that is present in all cells. When the cell membrane of necrotic cells burst, LDH is rapidly released to the surrounding medium in proportion to the amount of dead cells. LDH activity can be detected in an enzymatic test described by Decker and Lohmann-Matthes (Decker and Lohmann-Matthes, 1988). Cell free media are incubated with nicotinamide adenine dinucleotide (NAD^+), which is reduced to $NADH/H^+$ by the LDH-catalyzed conversion of lactate into pyruvate. The catalyst (diaphorase) then transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), which is reduced to formazan. The colored product can then be measured spectrophoto-metrically at 490 nm.

TUNEL staining

Cell death in the aggregates (Paper I) was detected using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL). TUNEL is a technique where low molecular weight DNA fragments and single strand breaks ("nicks") are identified by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction catalyzed by TdT (Gavrieli et al., 1992). In our experiments, incorporated nucleotides were detected with a dUTP-specific, alkaline phosphatase (ALP)-conjugated antibody (ab) followed by adding the substrate nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphatate toluidine (NBT/BCIP). One advantage with the TUNEL staining is that it is not cell type specific. On the other hand, there is a graded intensity scale of stained cells, which complicates the interpretation of true apoptotic cells.

Fluoro-Jade staining

Fluoro-Jade (FJ) is an anionic, strongly acidic fluorochrome presented as a selective stain for degenerating and necrotic neurons (Schmued et al., 1997). However, it was recently reported that FJ also stain reactive astrocytes (Anderson et al., 2003; Colombo and Puissant, 2002). The exact mechanism for FJ staining is not known, but it is believed to be due to electrostatic interactions with a highly basic molecule (Schmued et al., 1997; Schmued and Hopkins, 2000). FJ stainings were used as a complement to the TUNEL stainings in Paper I and were performed as described by Schmued et al. (Schmued et al., 1997).

Nucleosome detection

In Papers II-IV, apoptosis was measured by detecting the amount of mono- and oligonucleosomes in cell lysates. As part of the apoptotic cascade, an endogenous Ca^{2+} and Mg^{2+} dependent endonuclease cleaves the DNA at internucleosomal linker regions (Nagata et al., 2003; Wyllie, 1980). However, the nucleosomal DNA is tightly complexed with the core histones and is therefore protected from cleavage. Since DNA degradation occurs prior to plasma membrane breakdown, mono- and oligonucleosomes become enriched in the cytoplasm of the apoptotic cells. The nucleosomes were detected by the enzyme-linked immunosorbent assay (ELISA) based Cell Death Detection ELISA Plus kit from Roche using a biotin conjugated, histone-specific ab to bind the nucleosomes to streptavidin coated wells and a peroxidase conjugated anti-DNA ab for detection and visualization using the substrate tetramethylbenzidine (TMB) (Figure 12).

Apoptosis-related proteins

When cells generate mono- and oligonucleosomes they are at a late stage of apoptosis. In Paper IV, we wanted to follow earlier events of this process as well whereby some early apoptotic markers (p-JNK, p-c-Jun, and release of

cyt c) were detected. For further details, please see Figure 11 and the Western blot section on page 45.

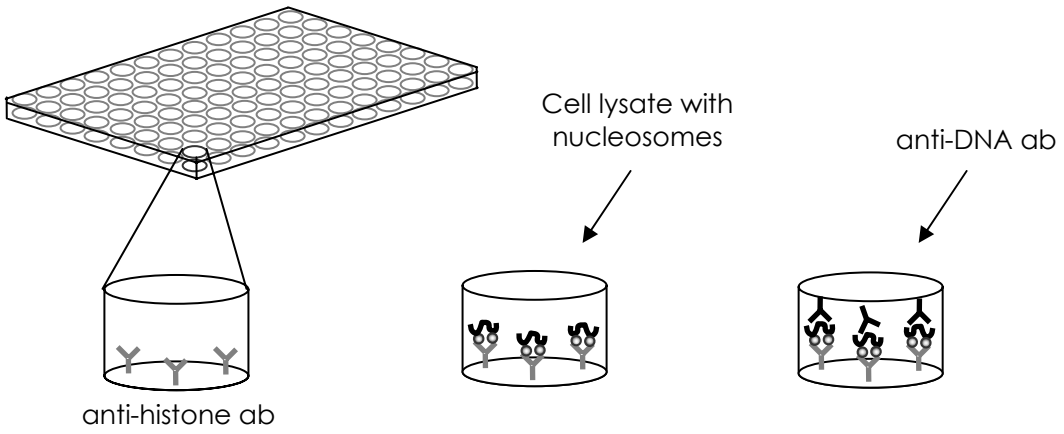

Figure 12: *Nucleosome ELISA principle. The nucleosomes, which contain fragments of DNA attached to histones, were captured to wells by histone specific ab's followed by detection with enzyme conjugated ab's specific for the DNA.*

CELL PROLIFERATION

Cell proliferation assays

In Paper I, non-EGF treated aggregates were analyzed for presence of proliferating cells by detection of proliferating cell nuclear antigen (PCNA) and 5-bromo-2-deoxyuridine (BrdU)-incorporation.

PCNA detection

PCNA is a DNA sliding clamp protein with many interactors, one function is to encircle the DNA template and prevent the replicative polymerases from dissociating while replicating the genome (Maga and Hubscher, 2003). PCNA is also active in the cell cycle where its expression increases substantially during the S-phase when proliferating cells synthesize their DNA. Even though the expression of PCNA increase in the S-phase, it is still present at low amounts throughout the entire cell cycle, therefore it may be difficult using this marker for quantitative analysis of proliferation as non-proliferative cells are partially stained as well (McCormick et al., 1993).

BrdU-incorporation

For comparison, proliferating cells were also detected by BrdU-incorporation. BrdU is a thymidine analogue, which can be incorporated in the DNA during the S-phase of the cell cycle and be detected by BrdU-specific ab's (Gratzner, 1982). In order to detect BrdU immunologically, the DNA needs to be denatured (Garrett and Guthrie, 1998). This was accomplished by treating the sections with 4 N HCl for 10 minutes.

VISUALISATION TECHNIQUES

Visualization of cells is often obtained by labeling proteins of interest with ab's conjugated to a fluorochrome or enzyme followed by detection of a fluorescent signal or a colored precipitate. Proteins can also be detected from cellular lysates or medium either by Western blot (size separation on gel) or ELISA (capture of proteins in a plate). However, information of the cellular location of the protein is then lost. Using electron microscopy, very small cellular structures such as organelles and synapses can be visualized. Sometimes sufficient information can be obtained by staining cells with unspecific dyes.

Cellular staining

Cell Tracker™

To morphologically distinguish THP-1 cells from SH-SY5Y cells in the co-cultures in Paper III, the THP-1 cells were stained with Cell Tracker™ Green 5-chloromethylfluorescein diacetate (CMFDA) (Molecular Probes) prior to initiating the co-cultures. Cell Tracker™ CMFDA is a colorless dye that is actively taken up by any viable cell. Inside the cells, cytosolic esterases cleave off the acetate groups resulting in a brightly green fluorescent product. At the same time, endogenous glutathione S-transferase reacts with the chloromethyl group (Zhang et al., 1992) making the probe cell impermeant, which avoid leakage to neighboring cells. At cell division, the dye is inherited by the daughter cells.

Trypan blue exclusion

To quantify live and dead cells, we used Trypan blue exclusion. Trypan blue is a cellular dye that is impermeable to intact membranes, but can enter dead or dying cells where the membrane is interrupted (Yuhas et al., 1974).

Protein detection

Aggregate processing

To visualize and quantify different cell types, proliferation, and apoptosis, the aggregates were preserved by formaldehyde fixation and embedded in paraffin wax. Formaldehyde is lipid soluble and passes through the membranes and cross-links free amino acid groups. The lattice work then holds the overall architecture of the cell together. In order to embed the aggregates, they were mounted in cylinder compartments made of PCR-tubes and transferred to permeable tissue holders (Histolab AB) (Figure 13). The paraffin embedded aggregates were sectioned at 4 or 30 µm in a microtome and mounted on microscope slides.

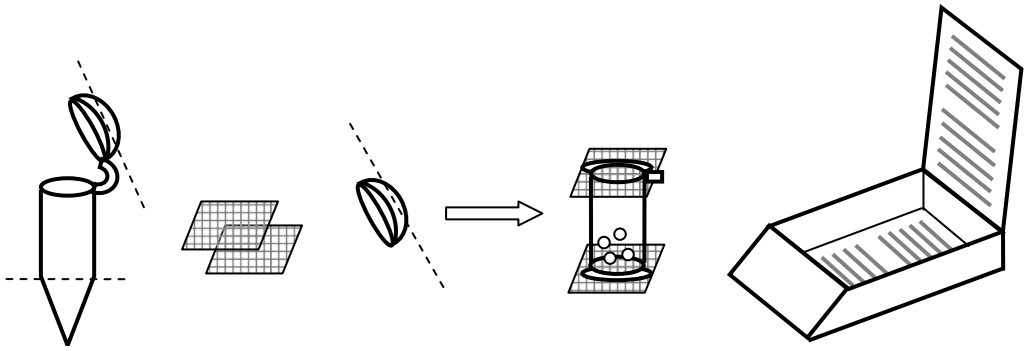

Figure 13: *Schematic illustration of aggregates mounted in PCR-tube derived cylinders flanked by pieces of nylon mesh. The cylinders were placed in permeable tissue holder cassettes prior to automatized dehydration and paraffin embedding.*

For detection of transmitter substances, neuropeptide Y receptor 1 (Y1R), and tyroxinhydroxylase (TH), cryopreservation of the aggregates was a more suitable alternative. Generating cryosections is faster than preparing the tissue for paraffin embedding. The drawback is that cryosections are less stable than paraffin sections and need to be stored frozen. When fixating the aggregates for cryopreservation, picric acid was added to the paraformaldehyde (PFA) to increase the penetration of the cell membranes and thereby improve the quality of the stainings. By adding sucrose in the buffers, the tissue was protected from ice crystal damage at freezing. The aggregates were embedded in mounting medium and snap-frozen. The embedded aggregates were cut in 12 µm thick sections, followed by thaw mounting onto microscope slides.

Immunohistochemistry

To visualize neurons in Paper I, we used an ab against the DNA-binding, neuron specific protein neuronal nuclei (NeuN) (Mullen et al., 1992; Sarnat et al., 1998; Wolf et al., 1996). NeuN is a nuclear antigen that is expressed early during development in neurons that has initiated terminal differentiation. However, there are specific groups of neurons scattered throughout the nervous system that, for unknown reasons, are non-reactive to NeuN (Sarnat et al., 1998).

Astrocytes were detected using an ab against the cytoskeletal intermediate filament GFAP, a marker commonly used for detection of astrocytes (Eng et al., 2000).

Many attempts were made to visualize oligodendrocytes using specific ab's, but with unsatisfying results. However, there are several published reports where aggregate oligodendrocytes and myelination have been a major focus (Copelman et al., 2001; Loughlin et al., 1997; Loughlin et al., 1994; Matthieu et al., 1978; Trapp et al., 1982). We tested several oligodendrocyte

and myelin specific markers such as 2, 3-cyclic nucleotide 3-phospho-diesterase (CNPase), proteolipid protein (PLP), MOG, and myelin-associated glycoprotein (MAG) on aggregates of different age. It has been reported that CNPase activity in aggregates increase at day 8 reaching a plateau around day 20-30 (Almazan et al., 1985).

To visualize MG we used two different markers, Griffonia simplicifolia B_4 Isolectin, which recognizes an a-D-galactosyl-bearing glycoprotein of the plasma membrane of macrophages/MG, and an ab against ionized calcium binding adapter molecule 1 (Iba1) (Imai et al., 1996). This macrophage/MG specific protein contains two calcium-binding domains (EF hands) that suggest involvement in calcium signaling. Iba1 is also involved in actin cytoskeleton regulation and MG activation (Imai and Kohsaka, 2002).

To avoid interference with endogenous peroxidase activity when detecting peroxidase conjugated ab's, mounted aggregate sections were treated in hydrogen peroxide (H_2O_2). Background was further reduced by blocking the sections in 5% serum from the species in which the secondary ab was generated.

Due to cross-linking, PFA fixation makes it difficult for some of the immuno-reagents to reach their target. The sections were therefore permeabilized with a detergent (Triton X-100). For some of the ab's, it was also necessary to uncover hidden antigenic sites by heating them in a protein stabilizing citrate buffer in a microwave oven (antigen retrieval).

Some of the primary ab's used in Paper I were generated in mouse (NeuN, PCNA, PLP, GFAP). To prevent increased background, we used the Mouse on Mouse (M.O.M.) kit from Vector, which efficiently block binding of secondary reagents to endogenous immunoglobulins.

To amplify the signals from biotinylated ab's on paraffin sections in Paper I, we used the Vectastain® Elite ABC-kit from Vector Laboratories, which contains an avidin:biotinylated enzyme complex (Hsu et al., 1981; Hsu et al., 1981). Due to binding of a high number of enzyme complexes to the biotinylated target, this detection method is very sensitive. We used an ABC-kit with peroxidase complexes and a mixture with the substrate H_2O_2 and the chromogen 3, 3'-diaminobenzidine (DAB) for visualization. The chromogen generates a brown precipitate that allows for resistant mounting in permanent organic-based mounting media (Mountex). The sections were counter-stained with Harris' Hematoxylin prior to mounting to visualize all cell nuclei.

In Paper I, we also detected proteins related to neuronal functionality, such as neuropeptides and transmitters. For this we used cryosectioned aggregates. The antigens were detected by tyramide signal amplification (TSA), a catalyzed reporter deposition method (Adams, 1992). The secondary ab's were conjugated with horseradish peroxidase (HRP), which catalyses deposition of biotinylated tyramine to the tissue. The biotin deposits were then visualized using fluorescein (FITC) and Texas Red labeled streptavidin respectively. After immunostaining, the sections were thoroughly rinsed and mounted in a mixture of glycerol and phosphate buffered saline (PBS) (3:1) containing 0.1% p-phenylenediamine to reduce fading of the fluorescence (Johnson and Nogueira Araujo, 1981; Platt and Michael, 1983). The aggregate sections were analyzed by fluorescence microscopy.

After fixation in formaldehyde, the neurospheres that were adhered to cover slips and allowed to differentiate in Paper I were stained with ab's against neurons (βIII-tubulin), astrocytes (GFAP), and oligodendrocytes (CNPase). The specificities were detected by fluorescence-conjugated secondary ab's, followed by analysis in fluorescence microscope.

The neuron specific βIII-tubulin ab used in Paper I, was also applied in Papers II and III to illustrate the neuronal morphology and to distinguish the SH-SY5Y cells from the green fluorescent Cell Tracker™ stained THP-1 cells. A red fluorescent Alexa Fluor® 594 ab (Molecular Probes) was used to visualize the SH-SY5Y cells. All cells were also stained with Hoechst; a membrane-permeable fluorescent stain that intercalates with the DNA and serves as a nuclear marker.

Cell lysis

In paper I, we wanted to detect the expression of GFAP and the synaptic marker synaptophysin over time in aggregates by Western blot as a complement to immunohistochemistry. Aggregates were transferred to eppendorf tubes at 9 time points between days 2 and 42. The aggregates and cells were disrupted by sonication in a radioimmunoprecipitation (RIPA) buffer containing detergents that permeabilize the cell membranes and solubilize proteins. The buffer also contained a protease inhibitor to avoid protein degradation. Debris was spun down and the remaining supernatants, or lysates, were kept on ice or stored at -80°C until further analysis.

In paper IV, UV-treated or untreated SH-SY5Y cells were lysed in a 1% Triton X-100 buffer for analysis of phosphorylated proteins such as p-JNK, p-c-Jun and p-Bcl-2. The buffer also contained the serine-threonine phosphatase inhibitor NaF (Brautigan and Shriner, 1988) and the phosphothyrosine inhibitor sodiumorthovanadate (Na_3VO_4) (Swarup et al., 1982) to avoid dephos-phorylation. A Complete™ Mini EDTA-free protease inhibitor cocktail (Roche)

was also added to prevent protein degradation. The cells were kept on ice during the whole procedure and they were washed once in cold PBS prior to lysis. The cells were incubated for 5 minutes in lysis buffer followed by scraping and transfer to Eppendorf tubes. The lysates were stored at -80°C until further analysis.

For detection of cleaved caspase-3 in Paper IV, we used a 0.5% Triton X-100 lysis buffer containing 1% sucrose to prevent disrupture of the mitochondria and subsequent leakage of mitochondrial proteins that could interfere by activating caspase-3. Prior to lysis, the above mentioned protease inhibitor cocktail was added together with dithiothreitol (DTT), a reducing agent that prevents oxidation. The proceeding lysis was performed as described above.

In paper IV, we also wanted to detect cyt c release from the mitochondria to the cytoplasm in UV-treated cells to investigate involvement of the mitochondrial apoptosis pathway. Crude mitochondrial and cytoplasmic fractions were generated using a digitonin buffer (Budd et al., 2000). Digitonin forms complexes with cholesterol and other β-hydroxysteroids and therefore preferentially lysates the cholesterol rich cell membrane leaving the mitochondrial membranes intact. To optimize the digitonin buffer for SH-SY5Y cells, we added 0.01% Tween20 to generate the cytoplasmic lysates. By adding digitonin buffer with 0.05% Tween20 to the remaining cells followed by scraping, a mitochondrial fraction was obtained. This method of generating a mitochondrial fraction is presumably not as pure as harvesting mitochondria by differential centrifugation, but it does enable a separation of mitochondria and cytoplasmic fractions.

Protein assay

To allow for relative comparison of the total cell lysates in Papers I and IV, the total protein contents were quantified using the BCA Protein Assay Reagent kit from Pierce. This kit is based upon the biuret reaction where Cu^{2+} is reduced to Cu^{+} by protein in an alkaline environment. The presence of Cu^{+} is detected using a reagent containing bicinchoninic acid (BCA), which forms a purple-colored product with Cu^{+} that can be detected spectrophotometrically at 562 nm. By correlating the absorbencies with a protein standard, the total protein concentration in each sample was estimated. The protein content of the Tween/digitonin lysates in Paper IV was not measured as the digitonin lysis buffer contains EDTA and EGTA, chelators that bind Cu^{2+} and interfere with the assay. Also, DTT affects the proteins being a reducing agent.

Immunoprecipitation

In Paper IV, cyt c was enriched from the mitochondrial and cytoplasmic Tween/digitonin lysates by immunoprecipitation. The lysates were first pre-cleared using Protein A/G beads to reduce non-specific binding of proteins,

followed by adding beads and mouse IgG1 for removal of proteins that bind non-specifically to immune complexes. Finally, Protein A/G beads were added together with a cyt c-specific ab that captured cyt c. After centrifugation, cyt c was retrieved by reducing the pellet followed by Western blot analysis (Figure 14).

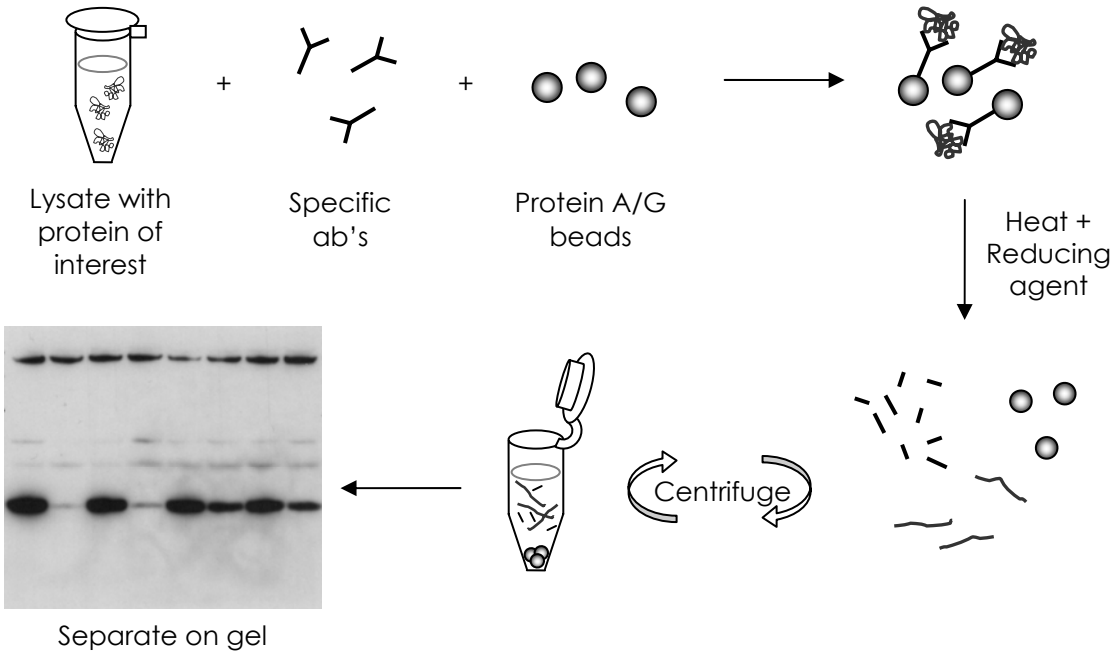

Figure 14: *Proteins can be enriched from lysates by immunoprecipitation where the protein of interest is captured by specific ab's that bind to Protein A/G beads. By spinning down the bead complexes, unbound proteins can be removed. To release the specific protein from the ab's and beads, the sample is denatured. The beads are spun down and the supernatant containing the protein of interest can be size separated on a gel for further analysis.*

Western blot

The Western blot technique was used in Papers I and IV to size separate equal amounts of total protein in the respective lysates. After transferring the proteins to membranes, potential background was reduced by blocking the membranes in milk. In the first round of protein detection, ab's against GFAP (Paper I), p-JNK, p-c-Jun, p-Bcl-2, cleaved caspase-3, and cyt c (Paper IV) were used. Secondary HRP-conjugated ab's were applied and the specific proteins were detected by adding ECL™ Western blotting detection reagent (Amersham Pharmacia Biotech) (In Paper I, the more sensitive ECL™ PLUS was used). HRP catalyses the oxidation of a substrate in the ECL™ solution whereby the product reacts with peroxide under emission of light that is captured on autoradiography film (Figure 15).

To allow for another round of protein detection using ab's with a different specificity on the same samples, the bound ab's were uncoupled by incubating the membranes in a mercaptoethanol buffer (Paper I) or

Restore™ WB Stripping buffer (Pierce) (Paper IV). In the second round of detection, ab's against the synapse-specific marker synaptophysin (Paper I), and the unphosphorylated forms of JNK, c-Jun and Bcl-2 (Paper IV) were applied and detected accordingly. In paper IV, a third round of detection was performed using an ab against β-actin. This served as an internal control for loading equal amounts of protein in each well.

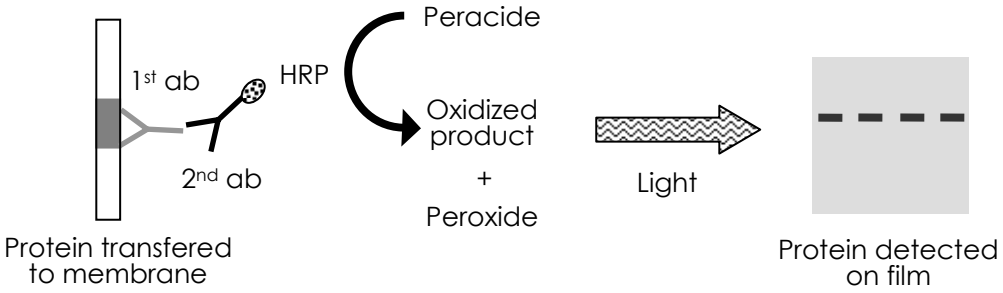

Figure 15: *Detection of proteins using the ECL™ Western blotting detection reagent.*

In situ hybridization

To verify the immunohistochemistry data obtained from the neuropeptide stainings in Paper I, we performed *in situ* hybridization. *In situ* hybridization is a method for localizing mRNA or DNA in cells or tissues using a labeled, complementary strand of nucleotides as a probe. mRNA is very sensitive for degradation by RNAses, which necessitates careful handling of the tissue. In our experiments, the probes were labeled with ^{35}S-dATP as previously described (Dagerlind et al., 1992). Sections (10 µm) of unfixed, frozen aggregates (4, 7, and 21 days old) were exposed to the radioactive probes. After hybridization, excess probe was washed away and the sections were coated in autoradiographic emulsion containing a silver salt that forms a precipitate after contact with light or radioactivity. After 7 to 31 days of exposure, the sections were developed and fixed. To simplify localization of positive signals to individual cells, the sections were counterstained with Toluidine blue prior to dehydration, degreasing, and mounting. The sections were analyzed by dark- and brightfield microscopy.

QUANTIFICATION TECHNIQUES

Densitometry

To quantify the amount of protein detected by WB, we used a densitometer that measures the density of the bands captured on the photographic film. In Paper IV, the density values were related either to the corresponding unphosphory-lated protein or to β-actin.

Stereology

In Paper I, the different cell types, including apoptotic and proliferating cells, were quantified in 4 µm immunostained paraffin sections using a 1 x 1 mm gridded ocular and a light microscope. Since some of the cell types were not distributed evenly in the aggregates, the accuracy of estimating the cell number using a grid on thin sections may be disputed on statistical grounds. Therefore neurons, astrocytes, MG, and the total cell number were also quantified using a defined and more rigorous methodology described below.

Optical fractionation

Optical fractionation is a stereological technique suitable for quantifying units in three-dimensional structures (Gundersen, 1986). Paraffin embedded aggregates were sectioned from top to bottom in thick (30 µm) sections (Figure 16 A) and each stack of sections was divided in three groups by random sampling. The sections in the respective groups were stained using cell specific markers (NeuN, GFAP, Iba1) (Figure 16 B). All sections were counterstained with hematoxylin to visualize the cell nuclei, which were consistently quantified from the NeuN-stained sections. Stained cells were counted within in a small volume of the section with a height of 10 µm covering an area (counting frame) that was individually adjusted for each marker (Figure 16 C, D).

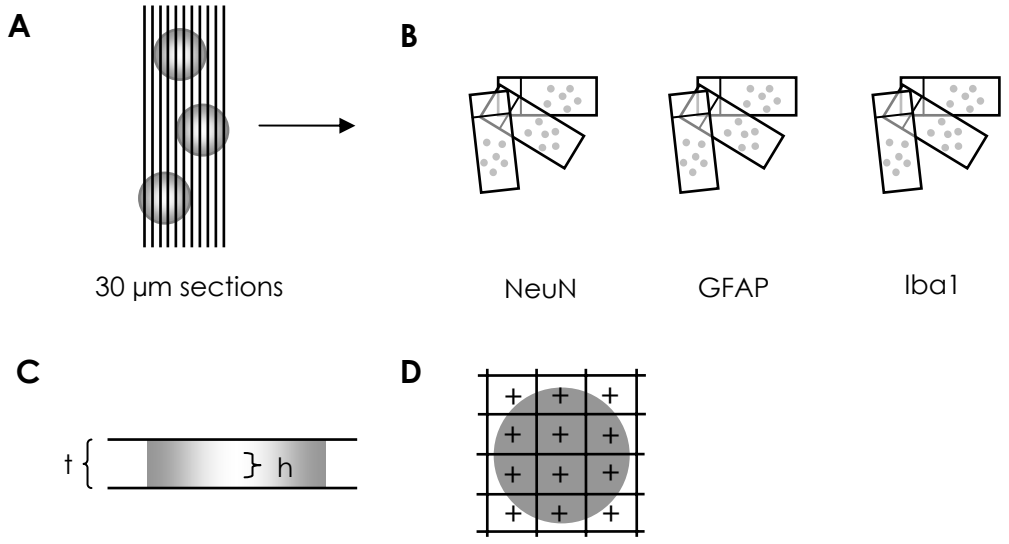

Figure 16: *The optical fractionation principle.* ***A*** *Paraffin embedded aggregates were sectioned in 30 µm thick sections.* ***B*** *Within each stack of sections, every third section was stained for a specific marker.* ***C*** *The number of stained cells was calculated in a height (h) of 10 µm within the section thickness (t).*
D *The sections were divided into fields (+) with counting frames.*

Both the counting frame and the number of counted fields/section were dependent on the frequency of the stained cell type (for high cell densities,

smaller and fewer areas were quantified). By counting several sections/staining, the number of cells within each aggregate was estimated.

Automated imaging

ImageXpress

In Papers II and III, the ImageXpress (IX; Axon Instruments, Inc.) automated cellular imaging and analysis system was used for quantification of apoptotic and necrotic cells, total cell number, and SH-SY5Y:THP-1 ratios. The IX allows for rapid collection (acquisition) of images from microtiter plates containing fluorescence stained cells. It uses the nuclear stain to focus and register each cell. The images are automatically analysed by the IX using cell specific algorithms designed to single out cells based on certain criteria. Cell debris was excluded by defining a cell area interval and apoptotic cells were distinguished from non-apoptotic cells by nuclear size and intensity.

STATISTICAL ANALYSIS

To assess statistical significance in Papers II-IV, Student's *t*-test was performed for single comparisons and a one-way analysis of variance (ANOVA) was applied for two or more comparisons. The ANOVA gives information about whether the different groups within the comparison differ from each other. To estimate the significance of the difference, a post-hoc analysis was performed. There are various post-hoc analyses available. We chose Tukey's, which is recommended if the numbers in each group of comparison are more or less equal in size. All experiments were performed at three separate occasions (n = 3) unless other stated in the original articles. The data was presented ± standard error of the mean (SEM) (Papers II-IV) or standard deviation (SD) (Papers II, III). Statistical significance was set to $P < 0.05$ for all experiments.

RESULTS AND DISCUSSION

CHARACTERIZATION OF AGGREGATE CULTURES (PAPER I)

In the 1950's, it was reported that dissociated cells from mammalian tissue spontaneously aggregate and re-form tissue-like clusters when cultured under suitable conditions (Auerbach and Grobstein, 1958; Moscona and Moscona, 1952). This culturing technique was greatly improved when Moscona, in the early 1960's, introduced rotation-mediated aggregation (Moscona, 1961). It was now possible to control the aggregate formation and size to obtain reproducible results, which facilitated comparisons between different species and tissues. This methodology of culturing re-aggregated cells under gyratory rotation has since then been modified by others to include mice, rat, and human tissue (DeLong, 1970; Garber and Moscona, 1972; Honegger and Richelson, 1976; Pulliam et al., 1988). The culturing conditions have also been further refined and optimized for serum-free medium, which allows for environmental control (Barnea and Roberts, 1999; Honegger et al., 1979). Aggregate cultures have proved to be a versatile *in vitro* system for studying complex mechanisms involving both neurons and glia. Examples of investigated areas are biochemical pathways (Honegger and Richelson, 1976), neuronal development (Barnea and Cho, 1993), neurotransmitter synthesis (Barnea et al., 1993; Honegger and Richelson, 1979), viral infections (Bale et al., 1987; Pulliam et al., 1984), myelination (Loughlin et al., 1997), and maintenance of neural progenitor cells (Berglund et al., 2004).

In Paper I, fetal mouse brain cell aggregate cultures were established from cortex and hippocampus derived from fetal mice and maintained in serum free, defined media for > 56 days. Using mice as model system has the advantage that it allows for studies using tissue from transgenic animals. However, the tissue volume per animal is reduced compared to for example rat. In general, aggregates derived from hippocampus and cortex behaved similar in culture except that the hippocampal aggregates continued to increase in size over time. Due to the limited access of hippocampal tissue from mouse embryos, we focused on characterizing cortical aggregates.

The aggregates underwent substantial proliferation and cell death during the first week in culture through which they stabilized in size and reached a final diameter of approximately 500 µm (even up to 1,000 µm) around day 9. Proliferating cells could be detected in the aggregates throughout the entire

culture period analysed (56 days). By immunohistochemical analysis of sectioned aggregates, we confirmed that the aggregates contained a mixture of neurons and glial cells. Quantification of the total cell number, neurons, astrocytes, and MG showed that among the specific cell types, NeuN positive cells (neurons) were generally the most abundant representing 20% of all cells at day 4 and approximately 50% at day 7, thereafter slowly decreasing to 25% until day 56. The number of GFAP positive cells (astrocytes) peaked between day 9 and 16, comprising about 10% of all cells whereas the number of Iba1 positive cells (MG) increased accordingly and represented 1% of the cells at day 16. In the developing rat brain, most neurons are generated during embryogenesis whereas the number of glial cells increases during the two initial postnatal weeks, which relates to the increase in glial cell number observed at day 16 (Frederiksen and McKay, 1988; Suzumura, 2002). A comparison with the cell composition in the adult rat cortex where approximately 40% of all cells are NeuN-positive shows that the aggregates contain a similar proportion of neurons (Herculano-Houzel and Lent, 2005). However, the majority of cells in the aggregates were not identified with the markers used (NeuN, GFAP, Iba1). The unidentified cells were probably a mixture of neurons and glia not yet expressing the markers used for detection. An interesting feature with the aggregates is that the cell ratios can be modified either by adding soluble factors or by introducing specific cells into the system (Loughlin et al., 1997; Loughlin et al., 1994; Pardo and Honegger, 2000). We found that replacement of the neuron promoting B27 supplement with G5, which favors glia, resulted in a larger proportion of glial cells.

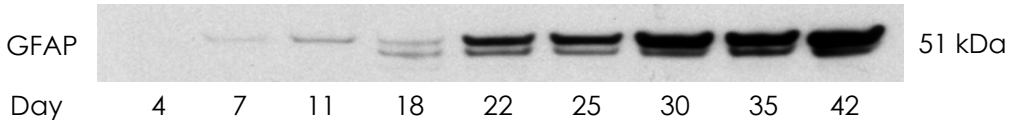

Figure 17: *In astrocytes, GFAP-expression increased over time in culture as detected by Western blot analysis of lysates generated at different time points.*

In young aggregates (< 14 days), the astrocytes were fairly small with thin processes. Over time they became more robust and the GFAP-expression increased (Figure 17), which correlates to observations made in the aging brain (Kohama et al., 1995; Wagner et al., 1993). Astrocytes were evenly scattered throughout the younger aggregates, however at later time points they were rarely found in the center of the aggregates probably due to increased aggregate density and impaired diffusion of nutrients (Figure 18 A). MG appeared evenly scattered throughout the aggregates at all time points generally presenting a rounded morphology with few and short processes. Maintaining MG in a resting state in culture is difficult as this cell type easily respond to environmental changes by activation. In mature aggregates (> 20

days), neurons were organized in a ring formation about two thirds from the center (Figure 18 B). The spatial distribution of neurons in aggregates has previously been explained as reconstruction of cortical layers (Garber et al., 1980). However, it could also be a consequence of nutrient supply. Another feature that changed over time was the expression of synaptophysin, a membrane protein present in synaptic vesicles (Jahn et al., 1985; Wiedenmann and Franke, 1985). We concluded that the increased expression correlates to an increased number of synapses. However, it does not necessarily indicate an increased synaptic activity in terms of neurotransmission as most components of the synaptic machinery are expressed long before differentiated synapses are established (Fletcher et al., 1991). Synaptic contacts have previously been identified in aggregates using electron microscopy (Garber et al., 1980; Trapp et al., 1979). In addition, the neurons expressed a number of functionally important neuropeptides and to some extent the corresponding mRNA, which indicate neuronal diversity. Barnea et al. has published many reports describing NPY expressing neurons in rat aggregates (Barnea et al., 1993; Barnea et al., 1991; Barnea and Roberts, 2001) whereas the synthesis, storage, and release of other neuropeptides has been described in detail by others (Honegger and Richelson, 1979).

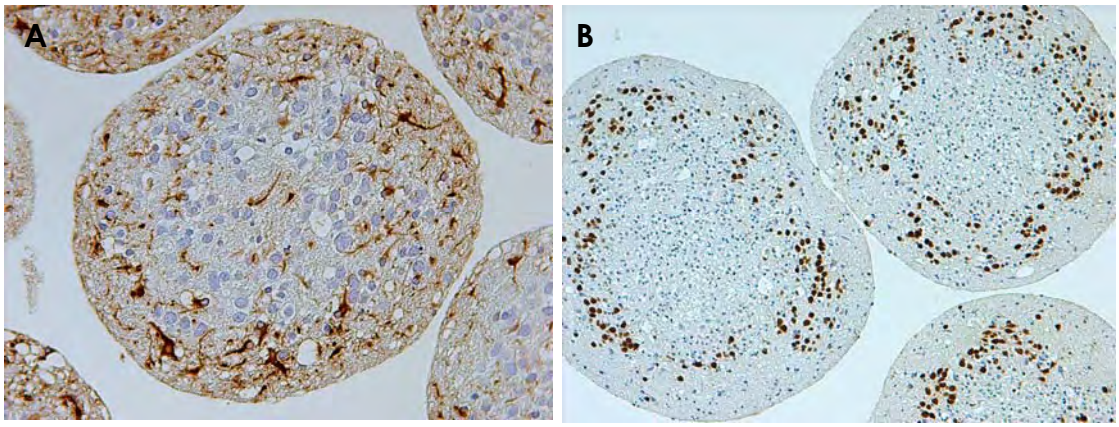

Figure 18: *Distribution of astrocytes and neurons in aggregates.* ***A*** *In older aggregates (45 days), astrocytes (GFAP) were rarely found in the center of the aggregates.* ***B*** *The characteristic distribution of neurons (NeuN) depicted in 25-days-old aggregates. (A=20x objective, B=10x objective).*

Since the aggregates were derived from fetal tissue it was reasonable to believe that they, apart from containing differentiated cells, also harbor neural precursor cells. To test this hypothesis, we added the mitogen EGF to cultures older than 7 days. EGF is known to facilitate proliferation, and inhibit differentiation, of precursor cells (Caldwell et al., 2001). Previous observations has shown that EGF-treatment of rat aggregates partially inhibit DNA synthesis and stimulate glial differentiation (Almazan et al., 1985; Guentert-Lauber and Honegger, 1985; Honegger and Guentert-Lauber, 1983). Our observation was

that the aggregate size, normally unchanged after day 9, significantly increased over time in the presence of EGF. Sections of EGF-treated aggregates revealed a layer of cells surrounding the original aggregate. The outer layer of cells was negative for GFAP and βIII-tubulin, but stained positive for nestin (Figure 19 A).

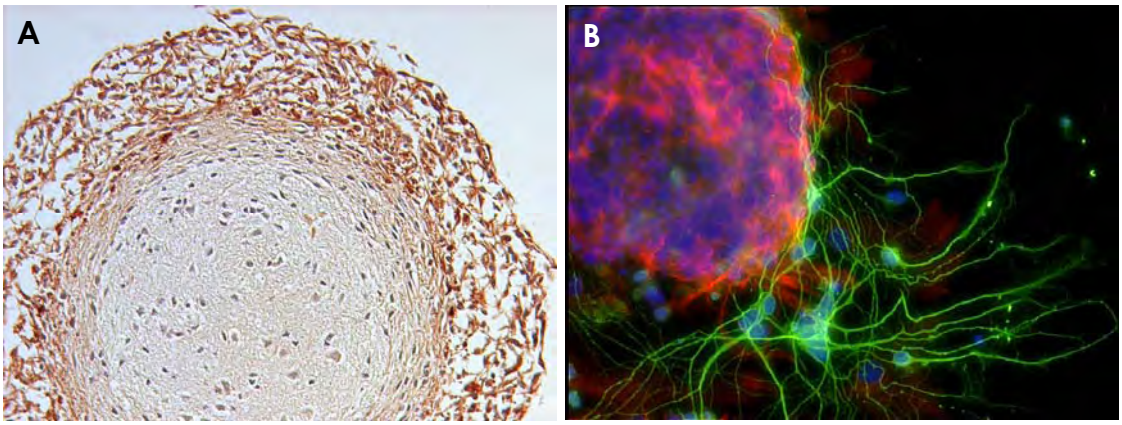

Figure 19: A *A 28-days-old aggregate cultured in EGF for 14 days to generate an outer layer of nestin-positive precursor cells.* ***B*** *A neurosphere, derived from dissociated aggregates, stained for neurons (green) and astrocytes (red). Nuclei are depicted in blue.*

Nestin is an intermediate filament protein that is widely used as a precursor cell marker based on its expression in neural progenitor cells and neuroepithelial cells during CNS development (Frederiksen and McKay, 1988; Lendahl et al., 1990). However, nestin is not considered a specific marker for neuronal precursors as it is expressed in differentiating myoblasts, endothelial cells, reactive astrocytes, and microglia as well (Kachinsky et al., 1994; Lin et al., 1995; Mokry and Nemecek, 1998; Yokoyama et al., 2004). To further investigate the nature of the EGF-responsive precursor cells, EGF-treated, and untreated aggregates were trypsinized and seeded as single cells. In the presence of EGF, some cells divided and generated spherical cell clusters, so called neurospheres. We observed that aggregates cultured with EGF generated larger quantities of neurospheres than the non-EGF-treated aggregates, which makes sense as the outer layer appeared to be rich in precursor cells. When dissociating the neurospheres, we were able to clonally expand single cells, a property that remained after several sub-culturings. Removal of EGF induced adherence of the neurospheres to a coated surface followed by differentiation into neurons, astrocytes, and oligodendrocytes (Figure 19 B). Thereby, we had shown that the aggregates contained neural precursors fulfilling the three stem cell criteria; self-renewal, clonal expansion, and multipotency (McKay, 1997). Neural precursor cells could be generated from aggregates even after two months in culture. EGF-treated aggregate cultures could therefore provide a competent source of neuronal precursor cells.

In summary, the fetal brain cell aggregates provide a dynamic yet organized three-dimensional environment for neurons and glial cells allowing for cell-cell interactions in a controllable milieu. The aggregates could be used as an in vivo-like system for studies where interactions and influence from various cell types are of importance. They could also be used for generation of neural precursor cells.

IMAGEXPRESS AUTOMATED IMAGING (PAPER II)

Recently, an increasing number of automated imaging techniques have been developed for rapid screening of cellular events (Li et al., 2003). Depending on the resolution, information from whole plates, single wells, cells or cellular compartments can be obtained (Ramm et al., 2003). High content biology has become increasingly popular especially regarding high throughput analyses and screening of compounds for the pharmaceutical industry (Perlman et al., 2005; Perlman et al., 2004).

In Paper II, we wished to evaluate the use of the IX automated fluorescence imaging system as a means to detect and quantify neuronal apoptosis. During apoptosis, neurons undergo several morphological changes, such as neurite retraction, disruption of the cellular membrane asymmetry, and nuclear condensation (Deshmukh and Johnson, 1997; Vermes et al., 1995; Wyllie, 1980), changes that can be used to discriminate apoptotic cells in a heterogenous population. A convenient and inexpensive way of detecting apoptotic cells by the IX is to incorporate a nuclear dye and detect the nuclear size in combination with its intensity. As described, apoptotic cells are characterized by nuclear condensation, which result in reduced nuclear size and increased nuclear fluorescence, so-called pyknotic nuclei.

To validate the system for detection of neuronal apoptosis, human SH-SY5Y neuroblastoma cells were cultured in 96-well plates and treated with different concentrations of the mitochondrial toxin rotenone for induction of apoptosis. The cells were subsequently fixed (PFA) and stained with Hoechst to visualize all cell nuclei. Images of the fluorescent cells were automatically acquired by the IX, which enabled rapid collection of a large number of images. These images were then automatically analyzed by soft ware algorithms. The analysis algorithms were initially designed to detect all cells based on cell specific criteria such as nuclear size and fluorescence intensity. Acquisition of images from a mixed population of normal and apoptotic cells followed by ungated analysis resulted in size and intensity plots where the populations could be distinguished and correct criteria chosen (Figure 20). From the nucleus size plot, a healthy cell was defined as having a nuclear size of 50-300 μm^2. The apoptotic cells appeared within a very narrow interval at around 75 μm^2 whereby the size of an apoptotic cell was set to 55-100 μm^2

(Figure 20 A). To increase the accuracy of the assay, a second criterion, nuclear intensity, was included. In the intensity plot, healthy cells appeared with nuclear intensities of 5,000-20,000 fluorescence units (Fl.U.) whereas intensities above 20,000 exclusively represented apoptotic cells (Figure 20 B). To assess neuronal apoptosis, the acquired images were analysed with analysis scripts based on the above criteria.

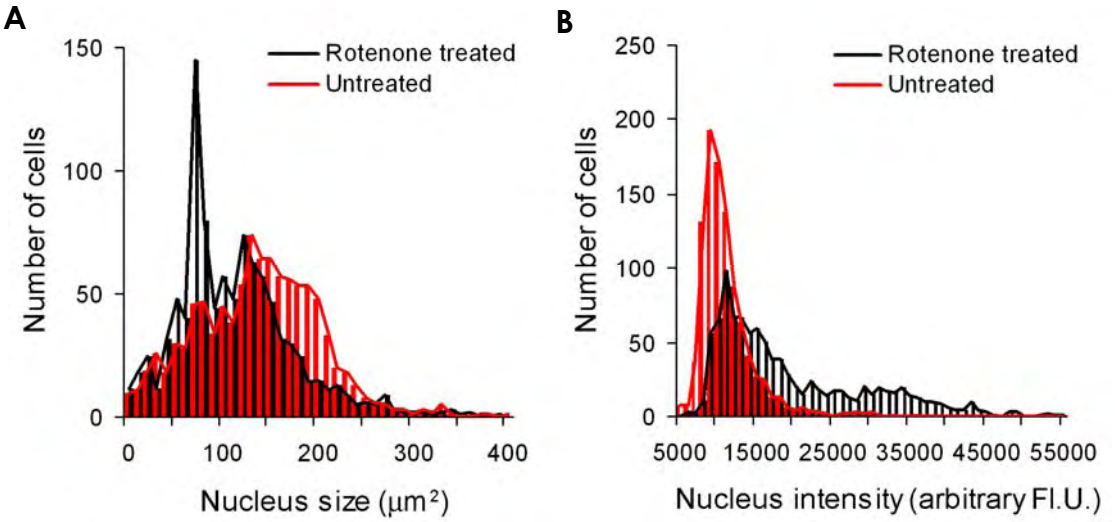

***Figure 20: A** Nucleus size and **B** nucleus intensity plots from 1,000 SH-SY5Y cells treated with 2 µm rotenone.*

Our results showed that rotenone dose-dependently induced neuronal apoptosis in SH-SY5Y cells, however at concentrations above 2 µM the amount of apoptotic cells detected decreased. To investigate whether this was due to loss of apoptotic cells from the plate during the fixation/staining procedures, we repeated the experimental set up, but measured apoptosis by detecting nucleosome contents in SH-SY5Y cell lysates. We obtained corresponding data with this method where cell loss is prevented by centrifugation prior to any removal of medium or lysate. We therefore concluded that the reduction in apoptosis at high concentrations of rotenone was a consequence of reduced proliferation as a response to the treatment.

To assess the IX assay stability, results from three plates seeded and analysed on separate occasions were compared. As a measurement of the inter assay stability, we calculated the EC_{50} value, which in our case corresponded to the concentration of rotenone needed to induce 50% of the maximum amount of apoptosis achieved in the assay. We found that while the EC_{50} values correlated well between the plates (Figure 21), the maximum level of apoptosis varied between approximately 14 and 24%. The inclusion of an internal positive control is therefore recommended for accurate comparison of samples between plates. The intra assay reliability was assessed by calculating the Z'-factor, which is a statistical parameter for comparison and

evaluation of the quality of an assay (Zhang et al., 1999). Z' range from 0 to 1 and values above 0.5 are used in high throughput screening assays to signify that an assay is of sufficient statistical separation to detect a random event with confidence. Z' was calculated in each of three plates and gave an average of 0.47. This Z' value is considered good quality for a cellular model. Additionally, the agreement we see in the Z'-factor between plates on different occasions agrees with the EC_{50} calculations demonstrating high inter assay stability.

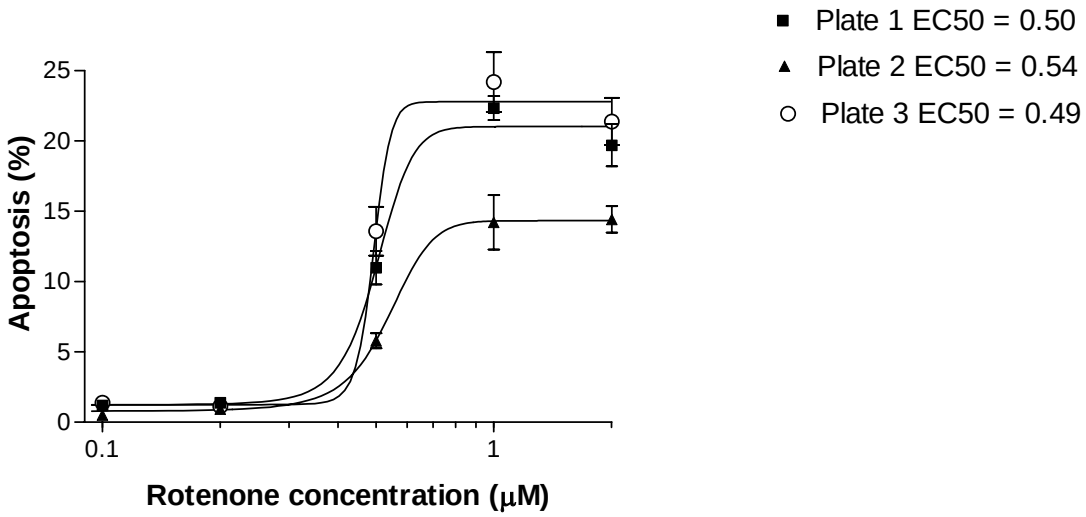

Figure 21: *Assay stability between plates (inter assay) was assessed by comparing EC_{50} for three plates from separate experiments.*

By adding the JNK inhibitor SP600125 to the cells prior to rotenone treatment, we observed that apoptosis was abolished. This is in agreement with a recently published study, which shows that apoptosis induced by rotenone is mediated via the JNK and p38 MAPK pathways in a caspase-dependent manner (Newhouse et al., 2004).

The great benefit from this method of detecting apoptosis is that the new systems to automatically image cells hinges on an initial detection of the nucleus. Thus our method can be built into all assays as a default. The IX automated imaging system is a versatile tool that, apart from detecting apoptotic cells, can be used as a means to detect and quantify neurite outgrowth (Orike, 2003) and to distinguish and quantify different cell types in a mixed culture (Aarum et al., 2003).

The IX proved to be a rapid and inexpensive high content screening assay suitable for morphological analysis of neuronal apoptosis in rotenone-treated SH-SY5Y cells. The IX can efficiently be used for screening of toxic substances and for titration of corresponding inhibitors. The IX also allows for generation of data on multiple parameters in cells stained with additional fluorescence markers.

MONOCYTIC EFFECTS ON NEURONS (PAPER III)

In this study, we wished to investigate the neurotoxic features of THP-1 monocytes. In particular, we aimed to further analyze the cell ratio at which effects could be seen. THP-1 cells in combination with SH-SY5Y neuroblastoma cells has previously been used as a model system for studies of monocyte-induced neuronal toxicity (Klegeris et al., 2005; Klegeris and McGeer, 2003; Klegeris et al., 1999).

THP-1 cells were used either unstimulated or stimulated with LPS and IFN-γ alone or in combination. After 24h, the THP-1 cells were either cultured separately or added on top of SH-SY5Y cells for 24h, 48h, or 72h at a seeding ratio of 1:2. In parallel, the corresponding CM were added to separate SH-SY5Y cultures. By measuring the amount of mono- and oligonucleosomes present in the cell lysates, we found that CM from LPS and LPS+IFN-γ stimulated THP-1 cells induced neuronal apoptosis at 48h and 24h respectively (Figure 22 A). Untreated or IFN-γ treated THP-1 cells did not produce enough neurotoxic factors to significantly induce apoptosis in SH-SY5Y cells.

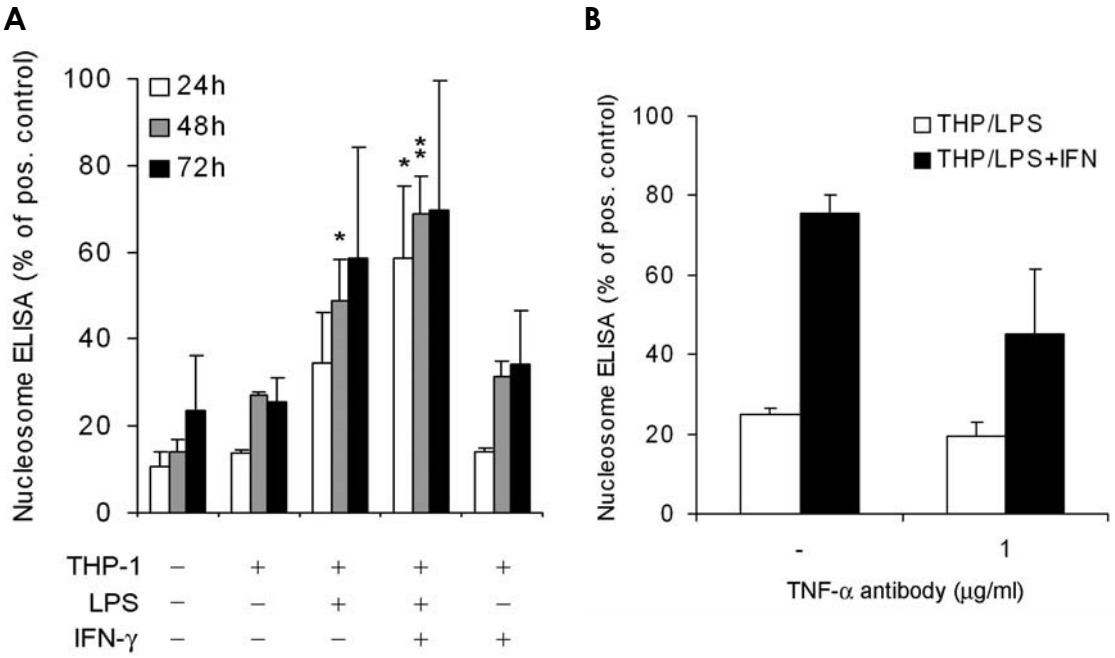

Figure 22: A *Conditioned medium from LPS and LPS+IFN-γ stimulated THP-1 cells induced neuronal apoptosis after 48h or 24h respectively, *P<0.05, **P<0.01.*
B *The neurotoxic effect of CM from LPS+IFN-γ stimulated THP-1 cells induced after 48h was reduced in the presence of a TNF-a neutralizing ab.*
Data in A and B are presented as mean ± SEM (n=3).

To investigate whether TNF-a could be involved in mediating the neurotxic effect of CM from LPS and LPS+IFN-γ stimulated THP-1 cells, we added a TNF-a neutralizing ab. TNF-a released by activated MG has previously shown to

mediate neurotoxicity in culture (He et al., 2002). We found that the TNF-α neutralizing ab decreased the neuronal apoptosis induced by CM from LPS+IFN-γ stimulated THP-1 cells with approximately 40% assessed by the nucleosome ELISA (Figure 22 B), indicating that TNF-α is involved in mediating the neurotoxic response together with one or several other factors. However, the TNF-α ab did not have any reducing effect on the neurotoxicity induced by CM from LPS-stimulated THP-1 cells. Another proposed neurotoxic mediator released by activated MG is ROS, we therefore applied the antioxidant Trolox to CM from LPS and LPS+IFN-γ stimulated THP-1 cells respectively. However, we did not see any change in the amount of apoptosis detected 48h later. Therefore, it is not likely that ROS are mediating the neurotoxic effects of CM from LPS and LPS+IFN-γ stimulated THP-1 cells in this system.

When co-culturing activated THP-1 cells in direct contact with SH-SY5Y neuroblastoma cells, we initially detected increased levels of apoptosis in the cultures where the THP-1 cells had been stimulated with LPS+IFN-γ. However, from the nucleosome ELISA assays, we could not specify whether the nucleosomes derived from apoptotic neurons or monocytes. In fact, the cultures that only contained THP-1 cells also indicated increased levels of nucleosomes. With Trypan blue exclusion, we could confirm that the LPS and LPS+IFN-γ treatment *per se* induced cell death in the THP-1 cells, an effect that previously has been reported in LPS-treated MG (Liu et al., 2001). To further investigate the neuronal condition after co-culture with stimulated THP-1 cells, we did a morphological assessment of the cells using the IX imaging system. Retraction of neurites is an early sign of neuronal injury (Deshmukh and Johnson, 1997), however, we could not observe any visual signs of neuronal injury or apoptosis in presence of THP-1 cells irrespective of prior stimulation (Figure 23). One possibility is that the neurons influence the THP-1 cells in a way that makes them less neurotoxic. In previous reports, neuron-glia interactions have shown to induce neuroprotective signals compared to CM (Zietlow et al., 1999). However, there are also reports where co-cultured MG exacerbates the neurotoxicity of certain neurotoxic compounds (Eskes et al., 2003). Another explanation could be that the toxic factors are released early after encounter with the stimulant. This means that these factors are present in the CM, but the expression may be downregulated after 24h when the THP-1 cells are added to the SH-SY5Y cells. It has been demonstrated that the secretion of TNF-α by LPS-stimulated MG peaks at 6h after induced stimulation (Liu et al., 2001).

Since the CM was generated from 1 x 10^6 THP-1 cells cultured for 24h in presence of the respective stimulant, the concentration of potentially neurotoxic factors in the medium exacerbated the amount of factors generated by the co-cultured THP-1 cells. To further investigate the

importance of sufficient concentrations of neurotoxic mediators, we serially diluted the CM to concentrations corresponding to the amount of factors generated in the co-cultures. In parallel, we also added higher densities of THP-1 cells to the SH-SY5Y cultures to see whether the absent neurotoxicity was a result of the cell ratio. We found that CM from LPS+IFN-γ stimulated THP-1 cells that had been diluted 1:4 induced significant levels of apoptosis in SH-SY5Y cells. However, the corresponding co-cultures did not indicate increased levels, which strengthen our observation that co-cultured THP-1 cells fail to induce neurotoxicity irrespective of prior stimulation.

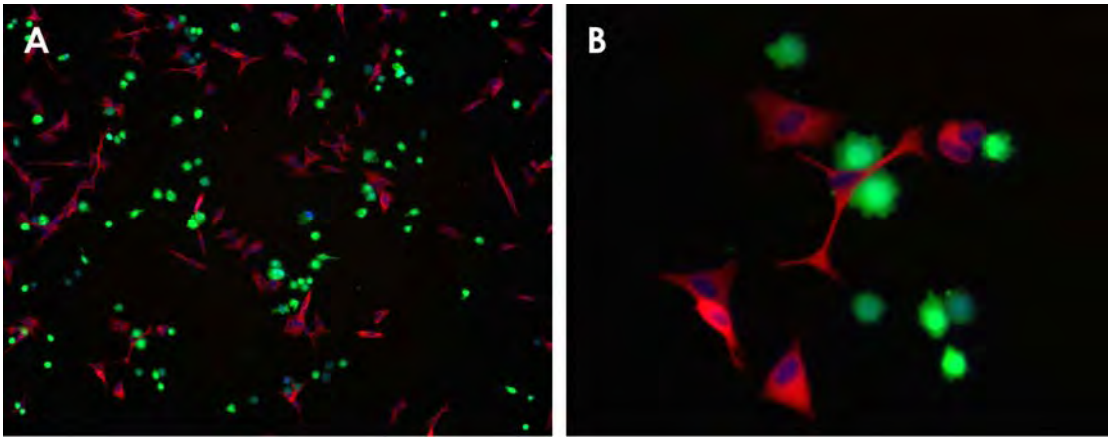

Figure 23: *SH-SY5Y neuroblastoma cells (red) co-cultured with LPS-stimulated THP-1 cells (green) for 24h. Nuclei are depicted in blue.*
A*10x objective* ***B*** *magnification from A.*

The monocytic/microglial effect on neurons is highly dependent on the degree of microglial activation, cell-cell contact, and the health of the neurons. Unstimulated MG has shown to be neuroprotective *in vitro* whereas activated MG impede neuronal survival (Zhang and Fedoroff, 1996; Zietlow et al., 1999). Activation of MG *in vitro* may also differ from activation *in vivo* thereby resulting in a modulated response towards the neurons (Hurley et al., 1999). Microglial CM may influence neurons in different ways, either by decreasing the number of primary neurons surviving in culture or by increasing the survival rate in neurons exposed to glutamate (Zietlow et al., 1999). In cases where MG are allowed to interact with neurons, mainly neuroprotective signals have been deteced (Zhang and Fedoroff, 1996; Zietlow et al., 1999). This is also the case for MG that have been activated by signals released from injured neurons (Polazzi et al., 2001; Watanabe et al., 2000).

We found that CM from LPS and LPS+IFN-γ stimulated THP-1 cells contain one or several factors, including TNF-a, that induce apoptosis in SH-SY5Y neuroblastoma cells. Culturing stimulated THP-1 cells in direct contact with SH-SY5Y cells did not result in detectable neuronal apoptosis. We conclude that the number of THP-1 cells needed to generate sufficient amounts of

neurotoxic factors exceeds any physiological ratio and that multiple factors are involved in determining the microglial effects on neurons.

UV-INDUCED NEURONAL APOPTOSIS (PAPER IV)

The initial aim with Paper IV was to generate a model system for induction of graded neuronal injury ranging from a mild insult to severe damage. The idea was to study the microglial response to injured neurons and to see whether the insult could be delayed, prohibited or even reversed in the presence of MG. However, as we evaluated the model system, it became evident to elucidate the apoptotic signaling pathways induced by UV irradiation.

As expected, exposing SH-SY5Y cells to UV irradiation induced apoptosis as well as necrosis in a dose-dependent manner, which we detected by measuring LDH release to the culture medium and nucleosome content in the corresponding cell lysates. However, in this system the LDH release may also be a consequence of secondary necrosis following the apoptosis. From these initial analyses, we found that mono- and oligonucleosomes were detected already at 4h after 10 seconds of UV treatment (Figure 24 A). To further investigate the apoptotic pathways following UV treatment, SH-SY5Y cell lysates collected 4h, 6h, and 8h after UV irradiation were analysed for the cleaved form of caspase-3. Cleaved caspase-3 could be significantly detected 6h after UV teatment (Figure 24 B).

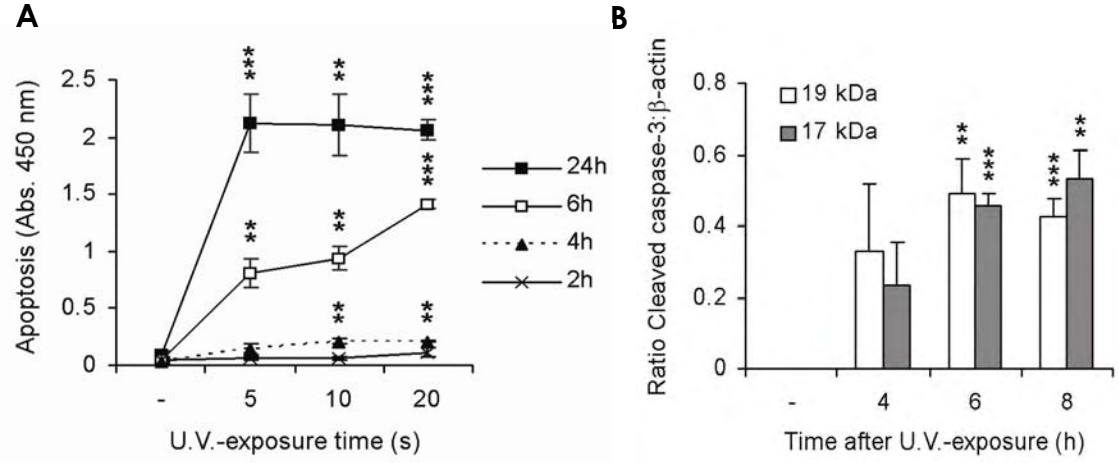

Figure 24: A *UV irradiation induced apoptosis and* ***B*** *cleavage of caspase-3 in SH-SY5Y neuroblastoma cells as detected by measuring the amount of nucleosomes in cell lysates and Wesfern blot analysis respectively. Data are means ± SEM (n=3), **P<0.05, ***P<0.001.*

Since the activation of executioner caspases is considered a rather late event, we continued by analysing potential upstream activators at earlier time points following UV.

The SAPK/JNK signaling pathway is known to be induced by cellular stresses including UV (Bode and Dong, 2003; Mielke and Herdegen, 2000). We therefore investigated phosphorylation of JNK (Thr183/Tyr185) following UV irradiation. We found that activation of JNK is a rapid event following UV exposure in SH-SY5Y cells, elevated levels were detected already at 15 minutes after 10 seconds of UV treatment (Figure 11). The JNK activation was transient, however p-JNK did not return to basal levels within the 6h analysed. The transcription factor c-Jun is one of many JNK substrates (Minden et al., 1994). JNK has been shown to phosphorylate c-Jun at Ser63/Ser73, residues that are considered essential for stimulation of c-Jun activity (Smeal et al., 1991). To investigate this further, we assessed p-c-Jun (Ser63) in UV-treated SH-SY5Y neuroblastoma cells. We found that c-Jun phosphorylation coincided with JNK activation although with a slightly longer duration (Figure 11). Hence, activation of JNK and c-Jun are two early events following UV irradiation of SH-SY5Y cells.

To further investigate the importance of JNK, we treated the SH-SY5Y cells with the JNK inhibitor SP600125 prior to UV treatment. In the presence of SP600125, the activation of c-Jun (Ser63) was inhibited up to 70% (Figure 25 A), whereas the amount of apoptotic cells was unchanged. These findings indicate that the UV-induced activation of c-Jun (Ser63) is not exclusively dependent on the JNK pathway in this system and that there may be other phosphorylation mechanisms or apoptotic pathways involved. Correspondingly, it was recently reported that induction of p-c-Jun (Ser63/Ser73) following cytosine arabinoside (ara-C)-induced apoptosis is JNK independent and proposed to be mediated by a CDK-like kinase (Besirli and Johnson, 2003). CDK1/2/4/5-inhibitors have also shown to significantly reduce apoptosis in UV-treated sympathetic neurons and differentiated PC12 cells (Park et al., 1998). To investigate whether any of the three CDKs, CDK1, 2, or 5 were likely candidates for the remaining levels of p-c-Jun following JNK inhibition, we applied the CDK1/2/5 inhibitor roscovitine, alone or in combination with SP600125 to the SH-SY5Y cells prior to UV irradiation. We found that roscovitine *per se* induced apoptosis, which is consistent with previous findings (Monaco et al., 2004; Ribas and Boix, 2004). However, this induction seems to be dependent on the mitotic status of the cells since roscovitine fails to induce apoptosis in terminally differentiated cells (Park et al., 1996; Ribas and Boix, 2004). Roscovitine did not further decrease the amount of p-c-Jun whereby we concluded that CDK1/2/5 are not significantly involved in c-Jun phosphorylation at Ser63 following UV irradiation of SH-SY5Y neuroblastoma cells.

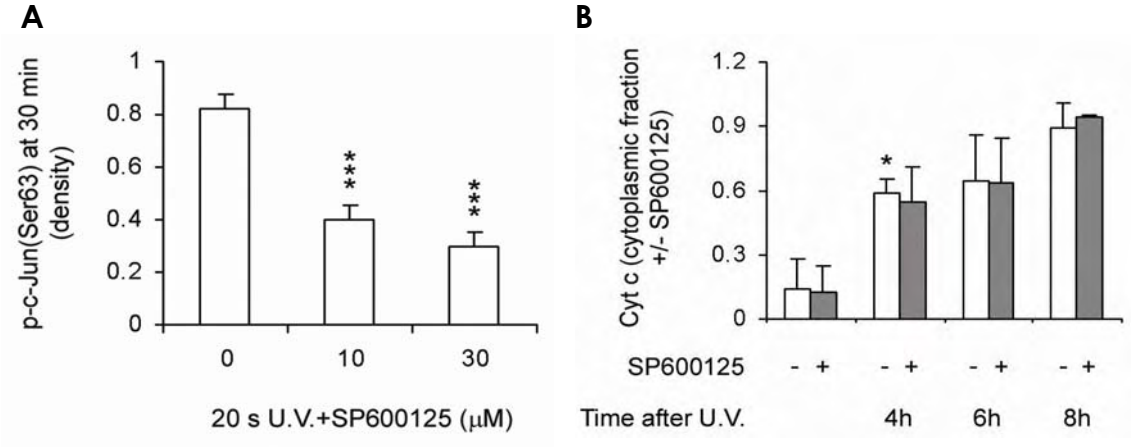

Figure 25: A *The JNK inhibitor SP600125 reduced phosphorylation of c-Jun with approximately 70%(30 μM) following UV irradiation.* ***B*** *SP600125 did not affect the release of cyt c to the cytoplasm following UV. Data are means ± SEM (n=4 and 3 respectively), *P<0.05, ***P<0.001.*

To investigate possible involvement of the intrinsic, mitochondrial apoptosis pathway, we measured the cyt c content of mitochondrial and cytoplasmic fractions of UV-treated SH-SY5Y cells. The results showed that cyt c was released from the mitochondria to the cytoplasm between 2 and 4h after UV treatment (Figure 25 B). Since JNK activation preceded cyt c translocation and reports propose that JNK is required for activation of the mitochondria-mediated apoptotic pathway after UV exposure (Schroeter et al., 2003; Tournier et al., 2000), we investigated cyt c release following JNK-inhibition with SP600125. We found that the cyt c translocation was not prevented by this inhibitor (Figure 25 B), neither was the activation of cleaved caspase-3 or as mentioned the ensuing apoptosis. We therefore conclude that factors other than JNK mediate the release of cyt c from mitochondria following UV-induced apoptosis in SH-SY5Y neuroblastoma cells.

Pro- as well as anti-apoptotic members of the mitochondria related Bcl-2 family of proteins are other proposed JNK substrates (Lei et al., 2002; Maundrell et al., 1997). However, it is still controversial whether phosphorylation of Bcl-2 reduces or enhances its anti-apoptotic properties (Ito et al., 1997; Yamamoto et al., 1999). We found that Bcl-2 was phosphorylated both in untreated SH-SY5Y cells and following UV exposure. However, no significant increase in p-Bcl-2 was observed. Incubating the SH-SY5Y cells with SP600125 prior to UV treatment reduced the levels of p-Bcl-2 (Ser70), a decrease that was not further diminished by adding roscovitine despite suggestions of CDK1-mediated phosphorylation of Bcl-2 (Furukawa et al., 2000). According to our results, neither JNK nor CDK1/2/5 was involved in mediating the phosphorylation of Bcl-2.

UV is a complex stress that can act on cells in many ways, not only by cross-linking death receptors with activation of the extrinsic apoptotic pathway as result but also by directly acting on mitochondria, inducing ROS or possibly causing release of cyt c via Ca^{2+} signaling (Pu et al., 2002).

We conclude that UV irradiation is a complex stress that induces neuronal apoptosis by engaging multiple mediators including factors involved in the mitochondrial apoptosis pathway and the JNK-mediated MAPK signaling cascade. We found that JNK is not essential for UV-induced apoptosis in SH-SHY5Y neuroblastoma cells and that other kinases than JNK may be invovolved in phosphorylation of c-Jun at Ser63/Ser73.

GENERAL CONCLUSIONS

In this thesis, I wished to investigate the neuromodulatory properties of MG using different culture systems ranging from complex co-cultures to more simplistic cell line models. Our initial aim was to study neurons and glial cells in a milieu that closely resemble the *in vivo* situation. We derived primary brain cells from fetal mice and by rotation-mediated re-aggregation of the neurons and glia, a third dimension of structural organization was added. This model allowed for intricate communication between the different CNS cell types, which is beneficial for neurons that are highly dependent on surviving signals from glial cells for their function and survival. The aggregate culture technique was entirely new for us when we established it in the lab. Characterization of the aggregates was therefore important for us to understand the benefits and limitations of the system in order to use it appropriately to address our questions. The aggregates were dynamic entities in which apoptosis and proliferation was substantial in the newly formed aggregates. Over time, the aggregates became more stable in size and cell contents showing an organized spatial distribution of the different cell types. The neurons increased their expression of the synapse-specific marker synaptophysin over time, which together with expression of important neurotransmitters indicated maturation and possibly generation of a communication network. Under our culture conditions, the aggregates contained a large proportion of neurons. However, this ratio could be skewed towards an increased number of glial cells. By adding a mitogen, we were also able to increase the proportion of neural precursor cells. This shows that the aggregates are dynamic and the contents can be modulated by external factors. The aggregates provided a suitable milieu for neural precursor cells, which makes the system usful for maintenance of neural precursor cells *in vitro*. One of the purposes using mouse tissue to generate the aggregates was to extend the use of these cultures to encompass transgenic mice. The comparison of normal aggregates and aggregates derived from transgenic animals could be valuable in elucidating the importance of certain factors or mechanisms in the developing brain. It would be interesting to futher characterize the aggregates in respect of functionality. Even though we have indications that synapses are formed, studying transmitter release or Ca^{2+} elevations would give more extensive information.

Although our aim was to study interactions between CNS cells in a milieu that closely resemble the *in vivo* situation, generating and maintaining the aggregates is rather laborious. The complexity of these cultures may be beneficial to address certain questions; however we found it too

complicated as a first model to study neuron-microglia interactions, especially on a molecular basis. We therefore decided to use a simpler model system to investigate the microglial effects on neurons in culture.

We started by investigating the proposed neurotoxic features of MG. As a model system we used SH-SY5Y neuroblastoma cells either cultured in CM from LPS and LPS+IFN-γ stimulated THP-1 monocytes or in co-culture with the corresponding THP-1 cells. The results from a cell line based system involving neuroblastoma and monocytes is quite different from the *in vivo* neuron-MG situation. However, MG in culture often resemble macrophages due to difficulties in maintaining MG in a resting state. Also, by stimulating monocytes with factors such as LPS and IFN-γ, they increase their expression of pro-inflammatory cytokines and up-regulate adhesion molecules and receptors becoming more macrophage-like. In this system, soluble factors released from LPS and LPS+IFN-γ stimulated THP-1 cells were sufficient to induce neuronal apoptosis. However, cell-cell mediated contact between neurons and stimulated THP-1 cells did not result in neuronal apoptosis as detected by nucleosome assay and by morphological assessment of neurons cultured in close vicinity of stimulated THP-1 cells for up to 72h. Since the THP-1 cells themselves contributed with nucleosomes in the co-cultures, the ELISA-based nucleosome assay may not have been optimal in detecting neuronal apoptosis. As discussed, it has been reported that the neuronal effect of MG, and monocytes/macrophages, is largely influenced not only by soluble factors released by neurons, but also by signals mediated by cell-cell contact. It is difficult to draw any valid conclusions from this system regarding the neuron-MG situation *in vivo*. However, we concluded that an unphysiological number of monocytes was needed to induce neuronal apoptosis and that the microglial/monocytic activators used in many *in vitro* systems over-activate the cells and induce apoptosis.

As discussed, injured neurons can induce microglial neuroprotection if the injury is not too severe. To study this further, we used SH-SY5Y neuroblastoma cells to work out a model for mild neuronal injury. We used UV irradiation to induce apoptosis because the cells can then be treated without replacing the medium after initiation of the injury. All factors relesed in response to the insult will therefore remain in the culture and co-cultured cells added after the insult would not be affected by the apoptosis inducing agent. We continued by investigating the time course of the apoptotic process by detecting activation of factors known to be involved in stress-induced apoptosis such as the JNK/SAPK pathway. We found that JNK is the primary kinase for c-Jun (Ser63/Ser73) although other kinases may be responsible for this activation as well. We could not see any significant involvement of CDKs, even thogh CDK-like kinases have been implicated in c-Jun phosphorylation in ara-C treated sympathetic neurons (Besirli and Johnson, 2003). We also

found that the release of cyt c is JNK-independent, which contradicts previous findings in UV-treated fibroblasts (Tournier et al., 2000). It is clear that UV is a complex stress that may induce apoptosis via multiple pathways and mechanisms, not necessarily identical in different cell types. It is also evidient that primary cells and cell lines can respond in different ways and that the type and severity of the insult can give different responses.

In vivo models are in many ways superior as a means to understand the mechanisms behind the complexity of an organism. However, these systems are complex and multiple factors cannot easily be controlled and may therefore interfere with the interpretation of the results. Studying cellular and molecular mechanisms in culture has the benefit of a reduced complexity and increased possibilities to modulate the environment compared to *in vivo* models. However, the information obtained from more simplistic systems must be interpreted with precaution in order to draw valid conclusions applicable to the *in vivo* situation.

SUMMARY IN SWEDISH

Centrala nervsystemet (CNS) är uppbyggt av ett intrikat nätverk av nerv- och gliaceller vilka är beroende av ömsesidig kommunikation för att utveckla och upprätthålla hjärnans funktioner. Om CNS drabbas av en skada eller sjukdom är det främst två celltyper, mikroglia och astrocyter, som reagerar genom att producera faktorer som kan hjälpa till att bekämpa infektionen eller att avgränsa det skadade området. Svaret från mikroglia kan dels vara positivt och främja nervcellers överlevnad, men det kan också vara toxiskt och orsaka skador på neuron och andra närliggande celler. För att närmare studera denna balans, och sedermera även celldöd (apoptos), användes olika cellodlingsmetoder som sträckte sig från komplexa primärkulturer till enklare cellinjer.

I första studien odlades hjärnceller från musfoster i kolvar på en skakapparat för att generera aggregat d.v.s. tredimentionella sfärer i vilka nerv- och gliaceller kan interagera med varandra. Aggregaten karakteriserades med avseende på cellinnehåll, viabilitet, uttryck av neurotransmittorer och respons på tillväxtfaktorer. Efter 4 dagar fanns det 20 gånger fler neuron än gliaceller i aggregaten. Detta förhållande ändrades över tiden och dag 16 utgjordes aggregaten av 1/4 gliaceller och 3/4 neuron. Denna prolifiering av gliaceller överensstämmer tidsmässigt bra med den tidiga ökning av glia som observeras under de närmaste veckorna efter födseln. Både nervceller och astrocyter mognade under odlingsperioden med avseende på ett ökat uttryck av synapsspecifika protein i neuron respektive filamentproteinet GFAP i astrocyter. Behandling av aggregaten med tillväxtfaktorn EGF inducerade proliferation av nestinpositiva celler vilka vi bedömde vara neuronala prekursorceller p.g.a. deras förmåga till regeneration, propagering och differentiering. Neuronala prekursorceller kunde utvinnas ur de odlade aggregaten efter mer än två månader i kultur. Aggregatkulturerna utgör en användbar modell som ligger mellan de enklare kontrollerbara cellsystemen och de mer komplexa *in vivo* modellerna eftersom de innehåller både nervceller och glia organiserade så att cellinteraktioner främjas samtidigt som odlingsförhållandena kan kontrolleras.

Närmare studer av interaktioner mellan neuron och mikroglia inleddes i en cellinjebaserad modell där SH-SY5Y neuroblastom odlades tillsammans med THP-1 monocyter. För att morfologiskt kunna bedömma huruvida monocyter inducerar apoptos efter att ha odlats tillsammans med nervceller, använde vi

ImageXpress. ImageXpress är ett automatiserat system där ett fluorescens-mikroskop kombinerats med en kameraenhet för att generera bilder av fluorescensmärkta celler odlade i en mikrotiterplata. För att validera detta system för detektion av neuronal apoptos inducerade vi nervcellsdöd m.h.a. toxinet rotenon. Behandlingen resulterade i kondensering av kromatin och förminskade cellkärnor, parametrar som användes i kombination med intensiteten hos fluorescensmärkta cellkärnor för att särskilja apoptotiska celler från normala celler. ImageXpress visade sig vara ett bra system för att detektera rotenoninducerad neuronal apoptos med avseende på dessa parametrar. Det visade sig dock att vi inte kunde detektera någon neuronal apoptos till följd av samodling med aktiverade monocyter. Som kontroll upprepades försöken men med skillnaden att apoptos mättes med en ELISA-baserad teknik. Detta bekräftade att aktiverade monocyter som odlats tillsammans med neuron under våra förhållanden inte inducerar neuronal apotos. Vi testade även att odla SH-SY5Y celler i medium som konditionerats av aktiverade THP-1 celler. Detta medium innehöll en högre koncentration av potentiella neuromodulerande faktorer än vad som kunde uppnås i co-kulturerna. Vi såg att konditionerat medium från LPS och LPS+IFN-γ stimulerade monocyter inducerade neuronal apoptos och att detta var dosberoende. Genom neutraliseringsförsök kunde vi se att neurotoxiciteten delvis medierades av faktorn TNF-a. Från dessa försök drog vi bl.a. slutsatsen att ofysiologiska mängder monocyter krävs för att inducera neuronal apoptos i detta cellsystem.

Slutligen ville vi undersöka om mikroglia kan förhindra skadade neuron från att genomgå apoptos, eller om de kan förhala apoptosprocessen. För att generera en neuronal skada där apoptos har inducerats men ej fullbordats följde vi SH-SY5Y celler som behandlats med UV-strålning över tiden. Vi såg att faktorerna JNK och c-Jun snabbt fosforylrades efter strålning och att denna aktivering följdes av caspas-3 klyvning och apoptos. Inhibering av JNK ledde till att fosforyleringen av aminosyrorna Ser63/Ser73 i transkriptionsfaktorn c-Jun minskade med 60%. Vi såg dock ingen minskning av cytokrom c frisättningen från mitokondrierna eller påföljande apoptos. Detta är en indikation på att JNK inte är nödvändig för att förmedla den UV-inducerade apoptossignalen i SH-SY5Y celler. UV strålning visade sig vara en komplex stressfaktor som kan aktivera flera olika mekanismer för att slutligen resultera i apoptos.

ACKNOWLEDGEMENTS

During my Ph.D. years, I have had the opportunity to meet and collaborate with lots of inspiring people that, together with my family, have supported me and guided me along the way both practically and mentally. I will therefore take this opportunity to express my sincere gratitude to the following persons:

My supervisor **Mats A. A. Persson**, for your generosity and big heart and for generating such a familiar atmosphere and good spirit in the MAAP-group.

My co-supervisor **Samantha Budd Haeberlein**, thank you for taking me under your wings! You are a true source of inspiration and encouragement. Thanks for sharing your enthusiasm and knowledge in neuroscience and the field of apoptosis.

My co-supervisors **Kristian Sandberg** and **Janne Näslund** for fruitful discussions and supervision during the aggregate years.

Tomas Hökfelt for sharing your incredible scientific knowledge, not only in the neuropeptide field, and for telling lots of fun anecdotes. **Katarina Åman** for teaching me *in situ* hybridization and for helping me out in the Hökfelt-lab.

Hans-Jürgen Gundersen for inviting me to your lab in Aarhus and for introducing me to the complex world of stereology. Special thanks to **Jens Nyengaard** for guiding me through the practicalities and for encouraging me to hang in there even when the nuclei turned into pink elephants ☺. Thanks also to **Maj-Britt Lundorf** for coming to my rescue complementing the counter stainings.

Jonas Frisén and **Niklas Holmström** for your introduction to stem cells and for demonstrating the neurosphere cultures.

The present and former members of the MAAP-group: **Johan Aarum** for sharing all those hours in the hood preparing our precious aggregates and for helping out with lab as well as computer related issues over the years. **Aster Beyene** for our thesis layout discussions and for teaching me how to run my first Western blot, it sure did come in handy ☺, **Katarina Drakenberg** for all your fun stories about Tiger, **Daniela Fernandez** for your supervision when I first

came to the lab, **Hannah Lindström** and **Marika Lundin** for making me a sushi addict ☺ and for initiating so many fun events for us to share outside the lab. Thanks also to **Tobias Allander, Ingrid Dinnetz, Kathrin Hopmann, Daniel Johansson, Ann Catrin Lagerkvist, Fayezeh Yari, Anila Yasmeen**, and to our e-bay promoter **Alexej Schmidt**. Also, a big hug to my first student **Marica Ekström**! Thank you all for creating such a wonderful working environment in the lab!

The groups of **Georgy Bakalkin**, **Tomas Ekström**, **Björn Johansson**, **Catharina Larsson**, **Gunnar Norstedt**, and **Lars Terenius**. Special thanks to **Sofia Johansson**, **Anna Ökvist**, **Daniel Edgar**, **Jesper Stockenstrand**, and **Annica Ericsson** for enjoyable lunch breaks and "fika".

Thanks to all the people in the sections of Bioscience and Molecular Science at AstraZeneca that I have had the opportunity to work with: **Jeanette Dypbukt** for your dissection lessons and for showing me how to find the "banana", **Jenny Bernström** for starting up the aggregate cultures with us, **Mia Nilsson** for your elaborate work to embed and slice the aggregates, **Dan Sunnemark** for sharing your skills in immunohistochemistry, and **Lena Appelsved** for helping out with practicalities in the lab. **Annica Rask** and the animal facility personnel for mating and taking care of our mice. **Anki Radesäter** for generously sharing your cell room at Novum, for all your help placing orders, sharing protocols, and for being my co-author. **Sara Johansson** for our enjoyable tour in the swamps of Louisiana, shopping and eating Beignets in New Orleans and for great company at Neuroscience 2003. **Lisa Peterson** and **Sergei Leonov** for sharing your THP-1 cells, **Anders Bäckstöm** and **Yvonne Nilsson** for always being so helpful, and **Kristina Hasslund** for your interest in my IX project. **Patriq Fagerstedt** and **Anne Jämsä** for sharing your office, **Anna Regner** and **Karin Agerman** for cell supply and nice chats. Thanks also to **Verena Wallentin** at Leed Discovery for sharing your SH-SY5Y cells and cell lab when I was in desperate need ☺. Finally, **Niklas Eriksson** and **Sverker Schäf** for helping out with UV cross linker transports and other practicalities. Thank you all for creating such a nice atmosphere in the labs at Novum and Södertälje.

All the members of the NIR-network for our enjoyable and educating meetings in Skåne and Tammsvik. Special thanks to **Mounira Djerbi** for being my roommate at Star Hotel and Örenäs. I have really appreciated your supporting talks and concern when times been tough. The network organizers and coordinators **Dick Heinegård**, the late **Kristina Sarvik**, **Mark Hickory**, and **Maria Olofsson** for putting your souls in making our network and meetings so fun and fruitful. Thanks also to **Dave Williamson** and **Janne Fellenius** at Wenell for your great team building courses and for teaching me how to stretch ☺.

"Saltis-gänget", **Anna Vetterlein** (and **Johan**), **Fredrik Carlsson** (**Jessica** and **Louisa**), **Jonas Jenner** (**Katarina** and "**Knytte**"), **Mikael Kullberg**, and **Michi Lundell** for great moments when we meet.

Peter Käll and **Helena** for your fabulous fall parties and **Henrik Jonsson** for willingly sharing deep thoughts.

Matt Gronski, thanks for your admirable attempts to teach me Polish and for thoughtful e-mails along the way.

Richard Warfvinge, for starting this roller coaster ride with me and for your support in the final loop.

All my newly encountered ancestors for many enjoyable hours at "Riksarkivet".

Mamma, tack för att "jag kan själv" och för att du alltid ställer upp, lyssnar och stöttar när det känns tungt och gläds med mig när det går bra (för det gör det ju trots allt ibland). Tack till **Lillebror** för att du finns och för att jag fick förtroendet att vara bilvakt under ditt Australienår, det gjorde resorna till Södertälje både roliga och snabba! En puss till **Musse** för att du är så klok och go! Morbror **Rolf**, tack för din omtanke och för att du engagerat dig i mina "råttor" ☺. **Mormor**, vad hade vi gjort utan dig? Med dina frysöppnings-haranger kan man komma långt även på labbet! **Farmor,** din förmåga att vara stark och och behålla humorn i mot- så väl som medgång har hjälpt mig mycket. Näst sist, men inte minst, en tanke åt min kära **Morfar** som håller ett öga på sitt gullehjärta varje dag, jag vet att du hade varit stolt över mig idag!

Finally, I would like to thank my älskling **Kåre Hultén** from the bottom of my heart for your belief in me and for your support along the ride. Thanks for putting up with me at all times and for sharing laughter and tears with me.

This project was supported by a grant from the "Network for Inflammation Research" funded by the Swedish Foundation for Strategic Research.

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