Phosphatidylinositol transfer protein α and its role in neurodegeneration

De rol van fosfatidylinositol transport eiwit α in neurodegeneratie

(met een samenvatting in het Nederlands)

Proefschrift

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Hanneke Bunte

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Promotoren: Prof. dr. K.W.A. Wirtz
Prof. dr. P.R. Bär

Co-promotoren: Dr. G.T. Snoek
Dr. F.L. van Muiswinkel

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"If brains were simple, we would be too simple to understand them".

Mario Puzo

Beoordelingscommissie:

Prof. dr. M.R. Egmond
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Dr. C.M. van Tiel

Paranimfen:

Els Bunte
Jan-Willem Jacquemijns

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Contents

Abbreviations

AA:	arachidonic acid
acetylated H_2DCF:	6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester)
AD:	Alzheimer's disease
ALS:	amyotrophic lateral sclerosis
BDNF:	brain-derived neurotrophic factor
CB1R:	cannabinoid 1 receptor
CMα/wt/c6:	conditioned medium of SPIα cells/ wild type NIH3T3 fibroblast cells/C6 astroglioma cells
CM16:	conditioned medium of C6 astroglioma cells in which PI-TPα is downregulated
CMscr:	conditioned medium of C6 astroglioma cells incubated with a scrambled RNAi construct that served as a negative control
CNS:	central nervous system
COX-2:	cyclooxygenase-2
DBB:	DMEM containing glutamax and 0.1% bovine serum albumin
DCF:	dichlorodihydrofluorescein
DGLA:	dihomo-γ-linolenic acid
ECS:	extracellular space
EPA:	eicosapentaenoic acid
GFAB:	glial fibrillary acidic protein
GPCR:	G-protein coupled receptor
H_2DCF:	6-carboxy-2',7'-dichlorodihydrofluorescein
H_2O_2:	hydrogen peroxide
HPLC/MS:	high performance lipid chromatography coupled to mass spectrometry
LA:	linoleic acid
LC/MS:	liquid chromatography coupled to mass spectrometry
LEα/wt/c6/16/scr:	lipid extract of CMα/wt/c6/16/scr
PARP:	poly(ADP-ribose) polymerase-1
PC:	phosphatidyl choline
PD:	Parkinson's disease
PI:	phosphatidylinositol
PIP_2:	phosphatidylinositol (4,5) bisphosphate
PI-TPα:	phosphatidylinositol transfer protein α
PLA_2:	phospholipase A_2
PLC:	phopholipase C
PL-TP:	phospolipid transfer protein
PNS:	periphery nervous system
RNAi:	RNA interference
SM:	sphingomyelin
SOD1:	Cu/Zn superoxide dismutase-1
SPIα/β cells:	NIH3T3 mouse fibroblasts overexpressing PI-TPα or β
TNFα:	tumor necrosis factor α

Chapter 1:

General introduction

In this chapter the relationship between neurodegeneration and the phospholipid transfer protein phosphatidylinositol transfer protein α (PI-TPα) is described. As the cellular organisation and function of the brain is a complex subject and a research field on its own, the first part of this chapter comprises an introduction to the different cell types that are present in the brain and the delicate balance between these different cells required for optimal brain function. In addition, several neurodegenerative diseases are discussed in which this balance is clearly disturbed. Finally, using the information presented in the first three paragraphs, the relationship between PI-TPα and neurodegeneration is explained.

1. History of Neuroscience

1.1. The Brain

The first written account about the brain and brain anatomy dates from the time of the ancient Egyptians. Figure 1.1 shows the hieroglyph for brain. However, until the time of the ancient Greek civilisation, it was thought that the heart was the central organ of sensation and thought.

Figure 1.1: Illustration of the hieroglyph used for the concept brain.

Alcmaeon of Croton (mid-fifth century B.C.), an Ancient Greek philosopher and medical theorist, is thought to be the first to consider that the brain was the place where the mind was located. The ancient Greek and Romans gained knowledge about the brain by dissection, however dissection was forbidden by the Roman Catholic Church during the Middle Ages.

Many facts of the macrostructures of the brain were resolved in the 1600s by the English and Flemish anatomists Willis and Vesalius. In 1791, using a dissected frog, Luigi Galvani showed that the brain functions by using electricity. Although later his experiment proved to be an artefact, his work has been an important first step towards modern understanding of the electrical basis of neural activity and the discovery of the membrane and action potential. In the 1800s, the work of, among others, Paul Broca (1862) and Karl Wernicke (1874) eventually helped to show that different areas of the brain serve specific functions. They showed that some

functions, such as speech and language, are usually controlled by a particular part of the cerebrum.
Contemporary with the interest in brain function, the (cellular) organisation of the central nervous system (brain and spinal cord) has gained attention and has been investigated in more detail.

1.2. The Neuron

In 1839, Theodor Schwann and Matthias Schleiden developed the cell theory [1] stating the concept that all organisms are composed of similar units of organisation, which they called cells (derived from the Latin word 'cellula', meaning 'small compartment'). At the same time the composition of the central nervous system (CNS) gained attention from histologists, cytologists, and pathologists.
During the nineteenth century there was an on-going discussion about the organisation of the CNS. One group of researchers, the 'reticularists', argued that the CNS consisted of a network tissue (or reticulum) formed by fused nerve fibers. The other group, called the 'neuronists', believed that the CNS consisted of distinct elements or cells.
Both groups used the same methods and material to prove their theory, but came to different conclusions because of the poor magnification and resolution of the microscopes that were available at the time.
In the early days of brain research, the thoughts of Joseph von Gerlach were believed to hold for the composition of the CNS. Von Gerlach was a true reticularist. He believed that the small processes of nerve cells fused to form a network. Furthermore he assumed that the processes in the network rearrange to form nerve fibers which finally end up in nerves [2].
The first scientist to describe a nerve cell was Johannes Evangelista Purkyne [3]. He used an achromatic compound microscope and a microtome to obtain thin slices of brain tissue. He and others discovered 'corpuscles' in the cortex of the cerebral cortex that were situated in rows, containing a nucleus and 'tail-like endings' that disappear in the gray matter. This is a description of cells that were later named Purkinje cells, after their discoverer.
Using further improvements in microscopy, Otto Deiters [4] was the first to show that the neuronal cell body and its processes, which he named protoplasmic processes (later called dendrites) and the axis cylinder (axon), are continuous

(Figure 1.2, [5]). He believed that dendrites, but not the axons, of different neurons fuse to a continuous network.

Apart from the development of the microscope, another significant improvement was made by the discovery of methods that selectively stain distinct parts of nervous tissue.

While experimenting with photographic techniques, in 1873 Camillo Golgi treated fixed tissue with silver nitrate for varying lengths of time. A dense precipitate formed on 1-5% of neurons and glia (see next paragraph). The black reaction, as this treatment was called, visualised entire cells [6].

Despite the discovery of a technique that allowed that the CNS could be studied in far more detail, Golgi still believed that the nervous system consisted of a continuous network.

The first scientist who doubted the network theory was the Norwegian zoologist Fridtiof Nansen. In 1886 he stated that he found no evidence of a network between nerve cells [7]. In the same year, independently from Nansen, the Swiss embryologist Wilhelm His discovered that during the early development in human embryos nerve cells were not in contact. In addition, The Swiss psychiatrist August Forel in 1887 observed that degeneration in the CNS did not spread, but was restricted to the edge of the cell. Therefore he also disagreed with the network theory (reviewed in [8]).

However, it was the Spanish histologist Santiago Ramón y Cajal who stated that all

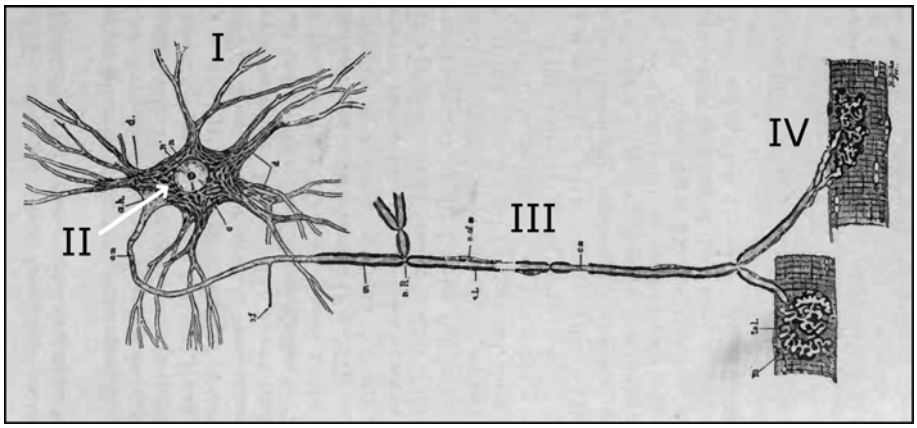

Figure 1.2: Illustration of a standard motor neuron adapted from Barker [5]. Processes marked with I were named protoplasmic processes by Otto Deiters [4] and later called dendrites. II points to the cell body. III represents the axon, which was named the axis cylinder by Deiters. IV shows the connection to the muscle (muscular end plate).

nerve cells were independent units in the CNS. He came to this conclusion by examining nervous tissue from different vertebrates. He improved the black reaction method of Golgi, allowing him to study tissue in more detail. In 1888, Cajal observed that the 'axis cylinder' of one cell ended close to 'protoplasmic processes' of another cell. This led Cajal to formulate the law of 'dynamic polarisation', according to how information runs in one direction through a nerve cell: from the protoplasmic processes through the cell body to the axis cylinder. As Cajal published in Spanish, his ideas did not get many followers until he attended a meeting in Germany in 1889. From that time on 'the neuron doctrine', as it was called later, gained more followers and in the end became fully accepted (Together with Camillo Golgi Cajal received the Nobel Prize in 1906).
The doctrine stated that:

- The fundamental structural and functional unit of the nervous system is the neuron
- Neurons are discrete cells which are not continuous with other cells
- The neuron is composed of three parts: the dendrites, axon, and cell body, and
- Information flows along the neuron in one direction

The terminology used above was only introduced in the last decade of the nineteenth century. The term neuron was introduced in 1891 by Vilhelm Von Waldeyer and was derived from the Greek word for tendon (reviewed in [8]). The axis cylinder was named axon by Rudolph von Kollicker and the protoplasmic processes were called dendrites by Wilhelm His. Sir Charles Sherrington described the 'gap' between the nerve and muscle and called it synapse in 1897 (composed from the Greek words 'syn' meaning together, and 'haptein', meaning to hold).
In the early 20th century the pharmacologists Harry Dale and Otto Loewi independently concluded from observations that neurons delivered information by secreting chemical substances that are now generally known as neurotransmitters.

1.3. Neuroglia

The pathologist Rudolph Virchow was the first to describe the space between neurons in the CNS. He stated that this space was filled with connective tissue, derived from the brain, covered by a layer of epithelium. He called this cement-like substance neuroglia (derived from the Greek word glia, meaning glue) and proclaimed that it appeared in the brain and spinal cord and embedded the neurons in these areas [9].

Cell staining techniques were only developed in the 1870s, therefore most of the studies were performed using unstained tissue that was carefully prepared, using fine needles to dissect the tissue apart in order to obtain single cells for investigation. For that reason, the retina was often chosen as study material, as this tissue is relatively thin and as a result less complicated to prepare.
In 1856, Heinrich Müller studied the retina and described radial fibers (later called Müller cells), which were, as he stated, non-neuronal cells [10]. Even though Müller's illustrations of non-neuronal cells in the CNS were convincing (Figure 1.3A, [11]), from a historical point of view Virchow is widely accepted as the scientist who first described non-neuronal cells in the CNS. As described in [12] he states that neuroglia contains cellular elements. The illustration in this paper is considered to be the first image of a non-neuronal cell (Figure 1.3B, [11]). In 1865, Otto Deiters was the first to publish illustrations of glial cells that bear a resemblance to what we now call astrocytes (Figure 1.3C, [11]) [4]. A few years later, Camillo Golgi noted that processes of glial cells contact blood vessels, which is characteristic for astrocytes. He also described glial cells which are located in groups or rows and whose processes were attached to nerve fibers (Figure 1.4, [11]). These are clearly features of cells later named oligodendrocytes [13]. However, from these data he failed to conclude that neuroglia consists of different

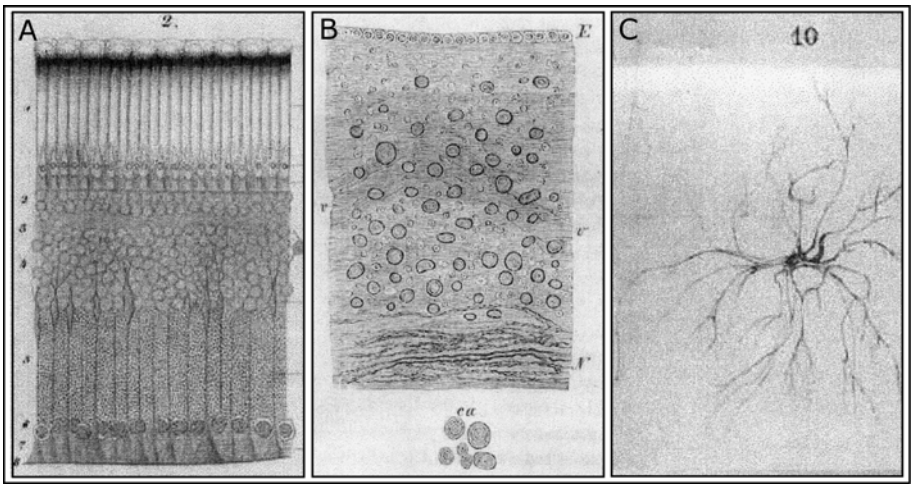

Figure 1.3: A: Illustration by Heinrich Müller, dated 1856, of radial fibers (Müller cells) in the retina. **B**: Image of glial cells, generated by Rudolph Virchow in 1858 (considered to be the first illustration of glial cells). **C**: Drawing of an astrocyte by Otto Deiters (1865). Figure A, B and C were adapted from [11].

cell types. Unlike Golgi, Michael von Lenhossék stated that glial cells were a mixture of different cell types and used the word astrocyte for the most common cell in neuroglia that is indeed star-shaped [14].

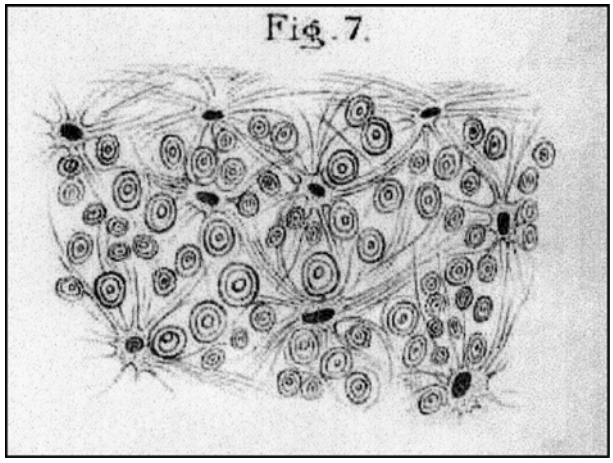

Figure 1.4: Oligodendrocytes visualised by Camillo Golgi. The processes originating from the oligodendrocyte cell bodies wrap around neuronal axons of which a cross section is visible. Image was adapted from [11].

In 1838, Robert Remak showed (Figure 1.5, [11]) that some nerve fibers in the peripheral nervous system contain a distinct cover and were thicker compared to 'normal' fibers [15]. Although these sheaths contained nuclei, it was first thought that they were secreted by axons. In 1839 Schwann confirmed Remak's observation [1].

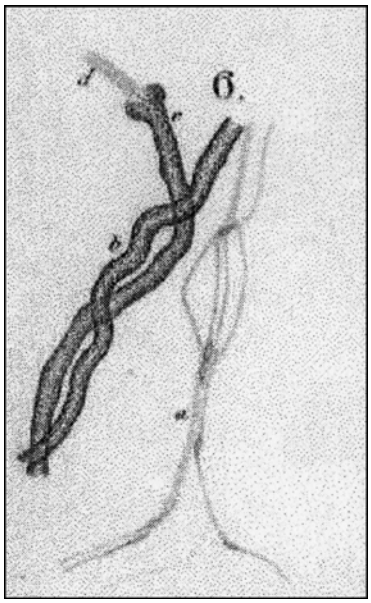

Figure 1.5: First illustration of a Schwann cell, visualised by Robert Remak. Image was adapted from [11].

Virchow first introduced the term myelin in order to refer to the fatty cover surrounding axons [12] and he, like Remak, believed that this substance was produced and secreted by the axons themselves. Although Remak was the first to describe these sheaths, they were called Schwann cells by Louis-Antoine Ranvier [16], who discovered that the coating around axons with Schwann cells showed interruptions. He therefore stated that an axon passes though a series of independent Schwann cells. These interruptions were named after him: nodes of Ranvier.

Santiago Ramón y Cajal stated that more types of glial cells than just astrocytes were present, the so-called third element of the CNS (i.e. the first and second element represent the neurons and astrocytes). His student, Pio del Rio-Hortega

developed a silver-carbonate staining [17], with which he selectively stained two different cell types. The first type, which was most common in white matter (parts of the brain containing mainly axons), he named oligodendrocytes [18]. This because these cells showed fewer processes compared to astrocytes. As oligodendrocytes showed resemblance in relationship to Schwann cells and myelin, he proposed that oligodendrocytes were the myelin-producing cells in the CNS.
Hortega also recognised microglia [19], as Nissl already did in 1899 [20] and named them the 'true third element', placing oligodendrocytes with astrocytes in the second element of the CNS. He stated that microglia originate from peripheral blood macrophages and that they can change from an inactive in to a phagocytic, active state in response to infection.
Although Golgi showed in 1871 that processes of glial cells (i.e. astrocytes) contact blood vessels and concluded from this that glial cells provide nutritional fluids from capillaries to neurons [13], Cajal did not agree with this theory and proposed that glial cells (i.e. astrocytes, as oligodendrocytes and microglia were not identified yet) serve as insulation for the passage of nerve impulses [21]. He also considered the filling theory of Weigert, who stated that glial cells serve a passive role in filling up spaces around neurons [22]. Although Cajal also disagreed with this theory, Weigert's idea has persisted a long time among scientists and is even under discussion today.

2. Physiology of the Brain

2.1. The neuron

Elements of all neurons reside in the CNS. However, many processes of neurons leave the CNS and enter the periphery nervous system or PNS to activate muscles or locate signals like touch, sound, smell or vision.
Neurons are typically composed of a cell body, with a fine axon emerging from one pole and a set of dendrites from the opposing pole (see Figure 1.2). This standard idea about the shape of neurons comes from the studies of Purkyne. However, the neuron is the most polymorphic cell of the body. Many types of neurons differ in size, shape and function. Thus, it is difficult to define if a cell is a neuron by a single feature like shape or location. Neurons form networks in the CNS and the periphery by contacting other neurons via synapses, i.e. small gaps

between a dendrite, axon or cell body of one neuron and similar structures of another neuron.

Neurons communicate via chemical and electrical synapses (the latter reviewed in [23]), in a process known as synaptic transmission. The fundamental course of action triggering synaptic transmission is the action potential. This is a propagating electrical signal that is generated by depolarising the membrane potential of the plasma membrane of the neuron. When this action potential is generated, it passes through in the direction of synapses located in dendrites at the end of the axon. There it induces the fusion of synaptic vesicles filled with neurotransmitter with the plasma membrane. Subsequently, the neurotransmitter is released into the synapse, where it diffuses to the post-synaptic neuron and binds to its specific receptor. The action of the neurotransmitter can be excitatory (e.g. glutamate) or inhibitory (e.g. γ-aminobutyric acid (GABA)) meaning that the activity in the target neuron may either increase or decrease, respectively. For example a motor neuron that functionally connects the motor cortex to a muscle in the leg, may release excitatory neurotransmitters to contract or inhibitory neurotransmitters to relax the muscle. In addition, the released neurotransmitter may activate receptors on the membrane of the pre-synaptic neuron thereby preventing the release of neurotransmitters and generating a negative feedback mechanism.

2.2. Neuroglia: The Astrocyte

Although in 1871 Camillo Golgi already suggested that astrocytes may provide nutritional support to neurons, it took decades before it was established that astrocytes and other glial cells served not only as brain glue. As the field evolved, it became clear that glial cells serve on one hand as support cells, and, on the other hand, are essential for optimal neuronal function. The illustration in Figure 1.6 shows how the different glial cells are localised with respect to neurons in the spinal cord.

Astrocytes are the most common glial cells in the brain and outnumber neurons by 1:10. These cells contain processes that contact blood vessels, and surround neurons and synapses. Astrocytes are coupled to each other by gap-junctions, which enables intercellular communication. Like neurons, astrocytes maintain a membrane potential that is sensitive to potassium ions. It is shown that astrocytes take up the excess of K^+ released by depolarised neurons, thereby maintaining a stable K^+ level in the extracellular space (ECS). This is important as K^+ in the ECS regulates transmitter release, cerebral blood flow, glucose metabolism and neuronal

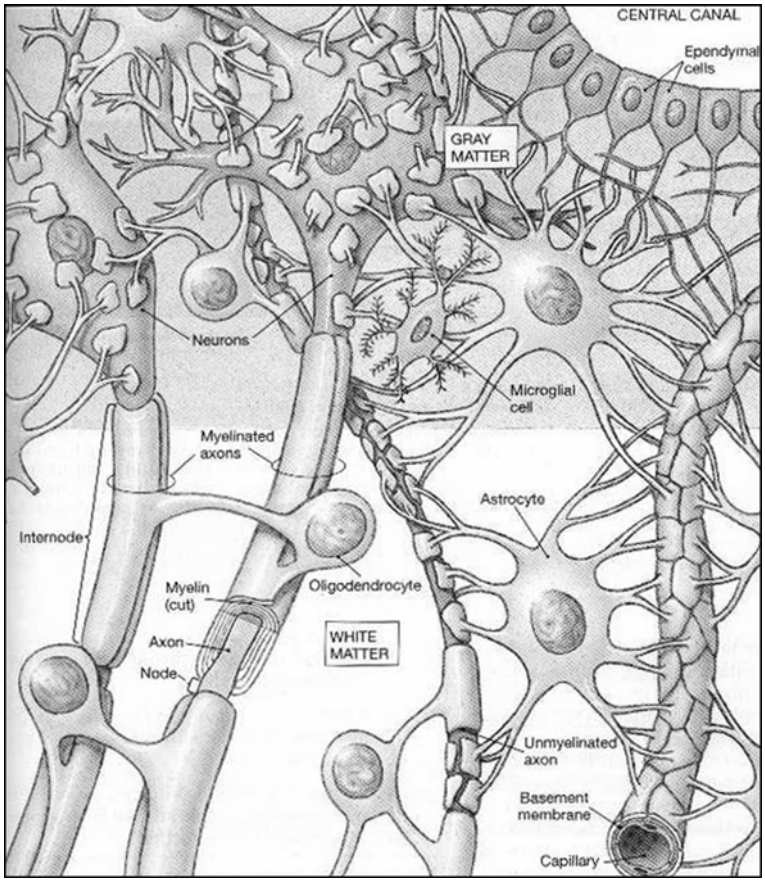

Figure 1.6: Cartoon of the cell composition in the spinal cord. The image shows neurons with their cell body in the gray matter and (myelinated) axons in the white matter as well as astrocytes, oligodendrocytes, microglia and ependymal cells (not mentioned in text)

activity [24]. Astrocytes are also responsible for the uptake of neurotransmitters like glutamate, the most common excitatory neurotransmitter in the CNS, after its release by neurons. This is necessary as remaining glutamate may overstimulate the neuron causing neuronal damage. This phenomenon is called excitotoxicity. Uptake occurs by glutamate transporters located in the plasma membrane of the astrocyte. In astrocytes, glutamate is converted to glutamine and transported back to neurons where it is transformed to glutamate and used again. This recycling system is called the glutamate/glutamine cycle [25].

By taking up glutamate, astrocytes sense neuronal activation. This stimulates glucose uptake by astrocytes from the circulation by glucose transporters located in

the plasma membrane of the processes that contact capillaries. Glucose is converted to lactate and transported to nearby neurons to serve as energy source [26]. Evidence is growing that astrocytes are also involved in plasticity and memory [27].

2.3. Neuroglia: The Oligodendrocyte

Oligodendrocytes are defined as the cells producing myelin sheaths that insulate CNS neuronal axons. In addition to myelinating oligodendrocytes, satellite and progenitor oligodendrocytes exist in the human CNS [28]. About the two latter types of oligodendrocytes, little is known. Myelinating oligodendrocytes however, have been investigated intensively. These cells produce sheaths of myelin (up to 1 mm wide) that wrap around axons of neurons in the CNS up to 40 times, thereby separating and insulating the axons. Myelination prevents transmembrane ion fluxes and thereby serves as an electrical insulator permitting rapid conduction of action potentials along the axon. The individual myelin sheaths are divided by nodes of Ranvier. Myelination of the neuronal axon increases the speed with which the generated action potential is able to pass through the axon, as membrane depolarisation is only required at the nodes of Ranvier. Myelin consists of two bilayers of plasma membrane, separated by a small amount of cytosol (cytoplasmic interface). The bilayers exist of lipids (70%) and proteins (30%), which is an inversion of the normal lipid-protein ratio in the cell body membrane of the oligodendrocyte that shows the normal 40-60% lipid-protein ratio. As myelin is in continuity with the cell body plasma membrane, a gradient must be formed [29] to accomplish this.

Myelinating oligodendrocytes show a slow mitotic rate and have poor regenerative capacity. Therefore they are the most vulnerable glial cells. Damage to oligodendrocytes therefore leads to demyelination. As a single oligodendrocyte cell is able to produce 20-70 processes of myelin that all wrap around different axons, degeneration of a single oligodendrocyte leads to disappearance of myelin segments around different axons. Remyelination is possible but occurs slowly and is often incomplete [30].

2.4. Neuroglia: Microglia

Approximately 5-12% of the non-neuronal cells consist of microglia. These highly mobile cells can be found throughout the adult CNS, but are of non-CNS origin. During embryonic development myeloid progenitor cells (which are also the

precursor cells for macrophages) enter the brain from the periphery and differentiate into microglia [31]. Thus, the microglial cell is the glial cell type that acts as the immune cell of the central nervous system. Normally, they exist in the CNS as quiescent cells, although recent investigations have demonstrated that 'quiescent' microglia exhibit active "immune surveillance" [32].
Upon injury of CNS tissue, microglial cells become activated. They change into macrophages, phagocytosing degenerating cells and debris and expressing a variety of hydrolytic enzymes. In addition cytokines and growth factors are synthesised and released.

2.5. Schwann cells

Schwann cells are the PNS-analogues of oligodendrocytes and therefore these cells are actually no component of the neuroglia cell types. They form myelin sheaths around axons of neurons outside the CNS. Schwann cells begin to form the myelin sheath in mammals during embryonic development and act by spiralling around the axon, sometimes with as many as a hundred revolutions. Unlike oligodendrocytes, myelinating Schwann cells provide insulation to only one axon.
Since each Schwann cell can cover about a millimetre of the axon, hundreds and often thousands are required to completely cover an axon, as axons in the PNS reach up to 1 meter. The individual Schwann cells are, like oligodendrocytes, separated by nodes of Ranvier.

3. Pathology of the Brain: Neurodegeneration

Neurons located in different parts of the CNS show different vulnerability and sensitivity towards endogenous and exogenous stimuli. As a reaction on such a stimulus, the delicate balance between neuronal and non-neuronal cells may be disturbed and, as a consequence, physiologically and/or anatomically related neuronal systems progressively degenerate. This is a hallmark of neurodegenerative disorders. As many different neuronal systems are present in the CNS, neurodegenerative diseases display a heterogeneous group of disorders. As most types of neurons do not divide, neurogenesis does not occur in many parts of the brain, and a degenerated neuron will often not be replaced. As a consequence, lesions develop and progressively the functions carried out by the degenerating part of the brain deteriorate.

Several commonly known neurodegenerative disorders are described below.

3.1. Alzheimer's disease

Alzheimer' Disease (AD) is the most common form of dementia in the elderly and is clinically characterised by cognitive impairment and changes in behaviour and personality. This is caused by the occurrence of (i) neuron loss, (ii) extensive loss of synapses and (iii) the formation of extracellular plaques (lesions) containing β amyloid protein as well as (iv) the presence of intracellular neurofibirillary tangles composed of aberrantly phosphorylated, insoluble forms of the microtubule-associated protein tau. These pathological changes occur mainly in the cortical and limbic areas of the brain [33]. However, what initiates the formation of the above described pathology is not yet fully understood. Hence, as a consequence, a treatment has not yet been found.

AD is a complex disease in which genetic as well as environmental factors may play a pivotal role. It is suggested that, among other factors, mutations in the genes encoding for amyloid precursor protein [34] and presenilin [35-37] are linked to AD. Environmental factors that have been associated with AD include accumulation of metals in the brain, traumatic brain injury, exposure to pesticides, and oxidative stress [38].

3.2. Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease after AD and is clinically characterised by slowness of movement, rigidity, tremor, and loss of postural reflexes. Pathological hallmarks include degeneration of dopaminergic neurons (neurons producing the neurotransmitter dopamine) located in the substantia nigra pars compacta. Surviving neurons show inclusions (insoluble protein aggregates) known as Lewy bodies containing neurofilament proteins [39].

The cause of sporadic PD (the majority of the PD cases) is unknown, but several investigations point to a multifactorial aetiology, involving a genetic predisposition (eg. polymorphisms on the apolipoprotein ε gene), environmental toxins, oxidative stress and ageing. In a minority of cases, PD is inherited. A number of associated genes, including α-synuclein (*SNCA*), parkin, leucine-rich repeat kinase 2 (*LRRK2*), PTEN-induced putative kinase 1 (*PINK1*) and *DJ-1* have been shown to cause PD [40].

At present, there is no cure for PD, but medication or surgery can provide relief of the symptoms. The most widely used form of treatment is L-dopa in various forms. L-dopa is transformed into dopamine in the brain. However, L-dopa does not prevent neurodegeneration and it causes a wide variety of side effects showing that the development of neuroprotective drugs is still required [41].

3.3. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS, commonly known as Lou Gehrig's disease in the US and motor neuron disease or MND in the UK) predominantly causes selective degeneration of motor neurons of the cerebral cortex (upper motor neurons), brainstem and spinal cord (lower motor neurons). Lower motor neurons connect the spinal cord to the voluntary muscles.

The loss of motor neurons leads to an irreversible progressive decline of muscular function, resulting in eventual paralysis, speech deficiency and, ultimately, death due to respiratory failure within two to five years after clinical onset of the disease [42]. Approximately 10% of the ALS cases are familial cases. Twenty percent of these patients (approximately 2% of the total cases) show a point mutation in the *ALS1* gene that encodes for the anti-oxidant enzyme Cu/Zn superoxide dismutase-1 (SOD-1) [43]. Other genes that are associated with ALS are the *ALS2-8* genes that are thought to encode for proteins responsible for organisation of the cytoskeleton (*ALS2*) and vesicle trafficking (*ALS2* and *ALS8*) [38].

The cause of sporadic ALS is unknown, but studies suggest an involvement of oxidative stress, excitotoxicity by glutamate, the presence of abnormal intermediate filament accumulations in motor neurons and an auto-immune response [44]. A cure for ALS has not yet been found. However the drug riluzole, which is thought to interfere with excitatory neurotransmission in the CNS by blocking glutamate release, is able to extend survival by approximately 3 months [45, 46].

3.4. Glial cells and neurodegeneration

In understanding the pathophysiology of neurodegeneration, the generation of transgenic and knock-out animals has been essential. Model animals for AD, PD and ALS have been developed and, together with analysis of post-mortem human material, they have revealed that, although different mechanisms are thought to contribute to neuronal loss in neurodegenerative disorders, the involvement of glial cells seems to be a common feature.

Among the first observed features following induced neuronal injury by e.g. acute trauma and ischemia [47] is the activation of microglia and astrocytes. In neurodegenerative disorders, this phenomenon often occurs in early stages of the disease. Sometimes even before actual neuronal loss.
Activated microglia show an increase in proliferation, increased and changed expression of surface antigens, migration towards damaged areas, and finally phagocytic action [48]. In addition, these cells secrete pro-inflammatory peptides, nitric oxide (NO) and excitotoxins that induce astrocytosis (astrocyte proliferation). More subtle activation of microglia however, can downregulate the neuroinflammatory process [49]. Upon activation by inflammatory factors or CNS injury, microglial cells are capable of secreting neurotrophic or neuron survival factors [50].
Morphologically, activated astrocytes exhibit hypertrophic cell bodies and the elaboration of distinct, long and thick processes. In addition, reactive astrocytes show increased expression of cytoskeleton proteins (e.g. glial fibrillary acidic protein, GFAP), cell surface and matrix proteins, proteases, protease inhibitors and growth factors and cytokines [51].
Debate is ongoing whether activation of glial cells after injury and during neurodegeneration is either neurotoxic or neuroprotective. Recently however, increasing evidence is provided for a neuroprotective role of activated glial cells [52].

In human post-mortem material of AD patients, proliferating activated astrocytes and microglia are associated with amyloid plaques. Astrocytes are thought to be activated by these plaques [48, 52]. Astrocytes are known to accumulate neuron-derived amyloid material, which leads to cell death of these, potentially beneficial, cells. In addition, tau protein is accumulating in astrocytes during the disease. Selective overexpression of tau protein has been shown to induce damage to neurons, including axon degeneration, although no significant neuronal loss was observed [53].
Reactive microglia found in the proximity of damaged areas of AD brain were found to stain positively for inflammatory-related compounds including complement proteins and immunoglobulin receptors [48]. This upregulation is associated with their role in phagocytosis of tissue debris. In addition the elevated secretion of inflammatory cytokines by reactive microglia may induce an increase in immunoglobulin (F_c) receptors, generating a autocrine positive loop [54].

These observations implicate that active microglia function as plaque-attacking scavenger cells as well as a source of highly cytotoxic substances [48].

In PD cases, astrocyte proliferation is also observed, which is thought to be associated with a neuroprotection mechanism. In addition nitric oxide production is increased. This may upregulate expression and secretion of the antioxidant glutathione, which is also observed. On the other hand, in microglia, the finding of upregulated expression of major histocompatibility complex (MHC) molecules emphasizes the notion that inflammation might be a characteristic of PD. Another feature found in PD is the increased levels of proinflammatory cytokines, which are thought to be secreted by microglial cells. Some of the detected increases however might be due to the chronic use of the anti-parkinsonian drug L-dopa [55].

Finally, in ALS patients, a strong astrocyte and microglial reaction is observed around upper and lower motor neurons that form a continuum along the damaged regions. Both cell types are activated and proliferate in these areas [49, 56].
Astrocytes show upregulation of inflammatory markers like cyclooxygenase-2 (COX-2) and inducible and neuronal nitric oxide synthase (iNOS and nNOS respectively). In addition, inclusion bodies and markers of oxidative stress have been observed [49]. A similar pattern of astrocytic changes has been described in the different mouse and rat models overexpressing human mutant SOD-1. In these animals the extent of astrocyte activation correlates with neuronal degeneration. Similar to other neurodegenerative diseases, protein aggregates are also detected [49].
It has been shown in ALS model mice expressing human mutant SOD-1 that treatment with minocyline, a compound that inhibits microglial activation [57], delays disease onset and extends survival in these mice [58].
However, besides evidence for a damaging role for activated glial cells in ALS, several studies show neuroprotective properties of glial cells in ALS.
Clement and co-authors [59] developed chimeric mice that expressed mutant human SOD-1 solely in glial cells or neurons. This study showed that normal motor neurons in the proximity of mutant glial cells develop ALS-like pathology. Concomitantly, normal glial cells significantly extended survival of motor neurons expressing mutant SOD-1, indicating that normal glial cells are capable of delaying neurodegeneration whereas introduction of mutant SOD1 in these cells induce

neurodegeneration. Concurrently, Beers et al. [60] showed that expression of human mutant SOD-1 in mice results in activated and neurotoxic microglia. Introduction of wild type donor microglia to SOD-1 mutant mice that were unable to develop microglia, slowed motor neuron loss and prolonged survival of mutant mice compared to mice that received mutant microglia or to normal SOD-1 mutant mice.

These data indicate that the above mentioned diseases are not purely neurodegenerative diseases. Glial cells, and in particular astrocytes and microglia, play an important role in different stages of the disease. Evidence is mounting that the majority of actions executed by these cells might be neuroprotective.

4. Neurodegeneration and PI-TP

As described in the former paragraph, upon certain, often unknown stimuli, related neuronal systems may undergo progressive neurodegeneration by as yet unidentified pathways leading to a decay of neuronal functioning. Glia-neuron interactions have shown to play an important role in this process. A specific protein that has recently been shown to be involved in neurodegeneration is phosphatidylinositol transfer protein α (PI-TPα).

4.1. Phospholipid transfer proteins

PI-TPα is a member of the phospholipid transport proteins (PL-TPs). These proteins were first discovered in membrane-free, rat liver cytosol [61] and have since then been identified in many different tissues derived from various mammals but also in plants, fungi and yeast [62]. Every protein molecule contains a single phospholipid-binding site. The distinction emanates from its affinity for a certain phospholipid headgroup or headgroups *in vitro*. Thus, PC-TP binds and transports phosphatidyl choline and the non-specific lipid transport protein (nsL-TP or sterol-carrier protein 2) binds and transports different phospholipids, as well as glycolipids and sterols [63].
The third class is named PI-TP as its mayor cargo is PI, although PI-TPs are also capable of transporting PC, while to a lesser extent.

4.2. PI-TPα, β, and homologues

PI-TP was first detected in membrane free cytosol of bovine brain tissue [64]. The protein responsible for the *in vitro* transfer activity of PI between membranes was classified to be PI-TPα in 1994. Its molecular weight was determined by SDS-PAGE and set to 35 kD [65]. Besides rat, PI-TPs have been shown to be expressed in all eukaryotic cell types investigated including yeast, fungi, parasites, amoebae, fish, fly and worm [66]. Among the different species the protein shows a remarkable sequence homology [62]. Table 1.1 shows the different homologues of PI-TPα that have been identified so far.

In the early 90s, a PI-TP domain (showing 41% sequence similarity to PI-TPα) was identified in an integral membrane protein in the retina of drosophila and named RdgBα [67, 68].

Subsequently, in 1994, an isoform was identified in bovine brain that was cross-reactive with antibodies raised against PI-TPα. This isoform, with a molecular weight of 36 kD, was named PI-TPβ [69]. Comparison of the rat brain cDNA of PI-TPα and PI-TPβ showed that the sequences were 77% identical and 94% homologous [70]. Despite this homology, major differences were found in their lipid transfer activity, their cellular localisation and protein expression level. As PI-

Table 1.1: characteristics of different phosphatidylinositol transfer protein (PI-TP) homologues

PI-TP homologue	molecular mass (kD)	sequence identity PI-TPα (%)	detected in	phenotype KO/KD	expression level	ref
PI-TPα	35	100	mammals	KO/KD: neurodegeneration and death in mice	ubiquitous; high in brain	[65]
PI-TPβ	36	77	mammals, worm, amoebae	KO: lethal *in embryo* in mice	ubiquitous; generally lower than PI-TPα	[69, 70]
PI-TP-like			drosophila (fly), parasite, worm, amoebae			[66]
(D)rdgBα	160	41	fly, worm	KO: retinal degeneration in fly	brain-specific	[67, 68]
(m)rdgBαI	160	44	mammals	fully restores rdgBα KO in fly	ubiquitous	[71]
(m)rdgBαII	147	46	mammals	partially restores rdgBα KO in fly	brain-specific	[72]
(m)rdgBβ	38	41	mammals, fly		ubiquitous	[73]
Sec14p	35	-	saccharomyces cerevisiae	deletions result in cell death		[74]

Abbreviations: KO: knock out; KD: knock down

TPα, PI-TPβ is able to transfer PI and PC between membranes *in vitro*. However, PI-TPβ also shows transfer activity towards sphinghomyelin (SM) *in vitro* [71, 72]. Whereas PI-TPα is mainly expressed in the cytosol and the nucleus, PI-TPβ was shown to be associated to the trans-Golgi complex [71]. As for the expression level, van Tiel and co-authors analysed several mouse tissues and showed that the expression level of PI-TPβ was between 15 (lungs) and 100 (brain and spleen) times lower when compared to the expression level of PI-TPα [73].
In yeast, PI and PC transfer are exhibited by the PI-TP homologue Sec14p. Like PI-TP, this protein has a molecular weight of 35 kD but shows no primary sequence homology with PI-TPα. For a fully covered review on sec14p, see [74].
The most recently detected homologue of PI-TPα is a homologue of RdgB, named RdgBβ [75]. This 38 kD soluble protein contains a small C-terminal domain and an N-terminal PI-TP domain and is found to be ubiquitously expressed in the cytosol.

4.3. PI-TP distribution in the brain

Besides RdgB, high levels of PI-TPα and β are also present in brain [76]. Using *in situ* hybridisation, the localisation of PI-TPα and β gene expression in developing and mature rat brain was determined [77]. As early as embryonic day 15 (E.15), both PI-TPα and β gene expression was detected throughout the brain and spinal cord. At post-natal day 0 (P0) PI-TPα and β expression was increased in gray matter throughout the brain. No significant expression was seen in white matter and in the cerebral ventricular zone. Concerning PI-TPα, the expression level remained similar on P7, whereas PI-PTβ gene expression was decreased slightly in some areas (i.e. the striatum, (hypo)thalamus, and brainstem). Between P7 and P21 a slight overall decrease of PI-TPα gene expression was observed in gray matter. Decrease in PI-TPβ gene expression was more pronounced, except in the cerebellar cortex.
On P35, PI-TPα expression was detected in almost all neurons and most prominent in cerebellar purkinje and granule cells. PI-TPβ expression was only found in the latter two cell types. No significant expression was detected in white matter. These results are roughly consistent with the data of Nyquist and

Helmkamp [78] on PI-TP (at the time these data were published, the existence of PI-TPβ was not yet known).
Using the same technique, in mature rats Imai and co-workers [79] confirmed high PI-TPα mRNA expression in several parts of the cerebellum, including purkinje cells. In addition, a high PI-TPα mRNA expression level was found in the brain stem, red nucleus and parts of the neocortex. An intermediate level was found in the hippocampus (CA3), thalamus, and motor neurons of the spinal cord. No hybridisation was observed in the olfactory bulb, basal ganglia, amygdala, hypothalamus and pituitary gland.
Strong mRNA PI-TPβ signals were obtained in the dentate gyrus. Moderate expression was detected in the olfactory bulb, layers of the neocortex, striatum, hippocampus (CA1-4) cerebellum, amygdala, hypothalamus, spinal cord, and pituitary gland. Low expression was found in the brain stem and thalamus.

4.4. PI-TP and neurodegeneration

Reduced expression of PI-TPα and its homologue RdgB have been associated with neurodegeneration in several organisms. Literature relating PI-TPs and neurodegeneration is summarised below.

4.4.1 RdgB null mutations in drosophila

Null mutations in RdgBα in drosophila result in light-enhanced degeneration of the photoreceptor cells in the retina [67] (hence, the name Retinal degeneration mutant phenotype B, RdgB). In mammals RdgBα occurs as two isoforms RdgBαI [80] and II [81]. Isoform I is widely distributed whereas isoform II possesses a neural-specific pattern with high expression in the retina [81, 82].
Mammalian RdgBαI and II and the PI-TP subunit of fly RdgB are able to rescue retinal degeneration in drosophila. However, while RdgBαI and the PI-TP subunit can also fully restore the electrophysiological light response, RdgBαII can only partially rescue the light response [81]. On the contrary, mammalian PI-TPα does neither repress degeneration, nor is able to restore the light response, indicating that PI or PC transport activity is not required for rescuing the phenotype [83].
The degenerative phenotype can also be repressed by raising the flies in the dark, which prevents retinal activity. Moreover, several additional mutations in other participants of the IP_3-mediated rhodopsin signalling cascade [84, 85], including fly phospholipase C (PLC) and a protein kinase C [58] or administration of a Ca^{2+}

blocker [86], could rescue the phenotype. Degeneration can be made light-independent by inducing mutations in a rhodopsin [87] or the Gα subunit that couples rhodopsin to the phospholipase C [88].
These data indicate that degeneration may not be caused by inositol phosphate signal transduction per se, since a second mutation in the inositol phosphate signalling pathway or a Ca^{2+} blocker can prevent degeneration. Sahly and co-workers showed increased Ca^{2+} spikes and hyperphosporylation in the RdgB null flies, and therefore suggested that loss of RdgB might cause Ca^{2+} toxicity by deregulation of downstream effectors of the PLC signalling pathway.

4.4.2 Reduced PI-TPα: the vibrator mouse

Mice developing spontaneous mutants, resulting in a distinct phenotype, offer an attractive approach to investigate the molecular pathway and cellular selectivity underlying that observed phenotype. Concerning neurodegeneration, the vibrator (vb) mouse showed an interesting phenotype. Homozygous animals (vb/vb) show a progressive, whole body action tremor when locomotion is initiated, starting from postnatal day 10-12. Weimar and co-workers [89] reported system degeneration restricted to neurons localised in the spinal cord, brainstem, cerebellum and dorsal root ganglia. This led to progressive motor paralysis, ataxia (unsteady motion due to a failure of coordination of muscle movements) and a tremor of cerebellar origin. The mice died of cyanosis due to respiratory failure within 40 days after birth.
Lifespan and the rapidity of functional deterioration showed to be highly dependent on the genetic background of the mouse strain, suggesting that the vibrator phenotype may be modified by other genetic factors.
The authors further reported that neurons that have not yet been degenerated show vacuolation of the endoplasmatic reticulum including the nuclear envelope. This rare cytopathological change is also documented in the lower motor neurons of the Wobbler mutant mouse [90], a well-characterised mouse model for motor neuron disease.
It was shown by Hamilton and co-workers [91] that the level of mRNA encoding PI-TPα in vb/vb mice was only 15-20% of the level in wild type mice due to a 6 kbase insertion into intron 4 of the *pitpn* gene. As a result, the level of PI-TPα protein expression was five-fold reduced in vb/vb mice, indicating that a low level of RNA was not compensated by increased translation or decreased turn-over. PI-

TPβ expression was shown to be unchanged when compared to wild type animals. Heterozygous animals showed 68% of wild type PI-TPα expression normalised to PI-TPβ and did not show any changes in phenotype compared to wild type animals. The authors also showed that neurofilament light chain was only weakly detectable in vb/vb compared to abundant expression in wild type animals. This was suggested to be a downstream effect of the vibrator mutation. In addition, the authors detected chromatolysis, a process in which the cell body starts swelling, ER-associated Nissl bodies diffuse and the nucleus is displaced. Because this is a reaction to axonal damage, the authors suggested that because of decreased PI-TPα expression, signalling in glial cells may be disrupted, which may lead to disregulation of axonal neuronal filament in neighbouring neurons, subsequently causing chromatolysis in the cell body. Other causes of neuronal degeneration in the vb/vb mouse put forward by [91], are Ca^{2+} toxicity, as similarly suggested for the RdgB null mutant flies, and blocked intracellular transport.
Monaco and co-workers [92] investigated the lipid content of brain and liver of vibrator mice. The authors found no significant changes in brain lipid content. In liver tissue, neutral lipid content was significantly increased in vb/vb mice. In addition, no alterations in lipid-mediated signalling were observed in prostatic fibroblasts obtained from affected animals. The authors suggest a possible role for PI-TPα in "some aspect of lipid intermediary metabolism".
Taken together it can be concluded from the data described above, that a reduced level of PI-TPα expression is associated with severe and fatal neurodegeneration in mice.

4.4.3 PI-TPα knock-out mouse

By injecting PI-TP$\alpha^{-/-}$ embryonic stem cells in normal blastocysts Alb and co-workers successfully generated PI-TP$\alpha^{-/-}$ knock-out mice [93]. At birth the mice are comparable to wild type mice, hence indicating that embryonic development is apparently normal. After birth however, 40% of the mice died within 48 hours. Mice surviving past day 1 (P1), show an increased rate of mortality between P2 and P12. No mice survived beyond P14. An actual phenotype started to develop from P4. The mice showed little spontaneous movement, but did respond to touch. In addition, the animals experienced seizures and an action tremor and were severely ataxic. As a result the mice were incapable of maintaining themselves upright. Moreover these mice failed to thrive. Body mass did increase, yet more slowly as

Table 1.2: Reported abnormalities in different organs of PI-TPα knock out mice [93].

Organ	Abnormality
CNS: Total brain	• Normal ATP level and ATP/ADP ratio • Increased levels of free fatty acid (FFA), sphingomyelin, lyso-PC and cardiolipin • Decreased level of 18:2(n-6) in triglyceride, diacylglycerol, and cardiolipin pools • Decreased level of 20:4(n-6) in triglyceride, diacylglycerol, cardiolipin, and FFA pools
Cerebellum	• Purkinje cells less branched, often misoriented • Reactive astrogliosis around neuronal cell bodies • ER deficits (vacuolations) • Decreased ATP level • Largely decreased ATP/ADP ratio
Spinal cord	• Thin and sparse ventral white matter • overall inflammation • motor neuron damage in ventral horn • apoptotic and aponecrotic* motor neurons • swelling of neuropil (non-neuronal areas) • demyelination
Brainstem and subthalamic region	• Increased astrogliosis
Perifery: Intestines	• Steatosis: accumulation of intracellular lipid in epithelium cells (enterocytes) associated with the ER
Plasma	• Decreased level of α-tocopherol (vitamin E), triglycerides, glucose, insulin, and glucagon • Increased level in β hydroxybutarate (measure for the activity of β oxidation in mitochondria) • Increase in corticosteroids (stimulus gluconeogenesis)
Liver	• Steatosis (reversible by fasting the mice) • Large increase of neutral lipid mass • Decrease in cardiolipin level • Aponecrosis* in hepatocytes • Decrease in ATP level • Large decrease in ATP/ADP ratio • Inappropriate glycogen stores
Pancreas	• Decrease in proglucagon expression • Decrease in absolute island number • Abnormal island morphology

* type of cell death associated with low energy level. For a review see text.

compared to wild type mice. In addition, the mice exhibited a low level of total body fat. This was not a result of suckling defects or dehydration.

Analysing post-mortem material of PI-TPα$^{-/-}$ mice revealed abnormalities in spinal cord and cerebellum including abnormal, apoptotic and aponecrotic [94] neurons, gliosis, and demyelination. In the intestines and liver abnormal fat

absorption and catabolism was observed. Finally the mice were severely hypoglycaemic. Reported abnormalities between PI-TPα$^{+/+}$ and PI-TPα$^{-/-}$ mice are listed in more detail in Table 1.2. Investigation of PI-TPα$^{-/-}$ embryonic stem cells [95], and embryonic fibroblast from PI-TPα knock-out mice showed that phospholipid metabolism was not changed when compared to wild type cells. From this the authors concluded that PI-TPα does not play a critical role in regulating phospholipid pools in most cell types and in PLC-dependent PI-signalling

Because ER derangement is commonly detected in PI-TPα knock-out mice (see also Table 1.2), the authors suggest that PI-TPα may be related to ER function, presumably in facilitating membrane trafficking at the level of budding of ER-derived vesicles.

According to the authors, selective degeneration of purkinje cells and motor neurons in the brain may be the result of the change in glucose homeostasis. As these cells are large and metabolically active cells, they might be more sensitive to insults. Decrease in energy levels by severe hypoglycaemia may result in the observed spinocerebellar disorder.

Ignoring the fact that the defects in the PI-TPα knock-out mice as described above are more severe and pronounced, the phenotype and biochemical abnormalities show remarkable parallels with the vibrator mouse. This underlines the importance of PI-TPα expression in the brain for neuronal survival

4.4.4 PI-TPβ knock-out

To analyse whether PI-TPβ exhibits any mammalian housekeeping function, Alb and co-workers [95] assessed the cellular consequences of PI-TPβ dysfunction by generating PI-TPβ null murine embryonic stem cells. However, although heterozygote cell lines were viable, the authors failed to identify PI-TPβ$^{-/-}$ clones. In line with this result, attempts to generate a PI-TPβ knock-out mouse were also unsuccessful. Strikingly, even PI-TPβ$^{-/-}$ embryos were never identified, indicating that the absence of PI-TPβ results in a developmental deficiency leading to embryonic lethality. In conclusion it appears that this protein is (at least) essential in embryonic development as well as cell viability.

4.4.5 PI-TPα and neurite extension

Recently, an additional neuron-related finding involving PI-TPα was reported [96]. It was shown that PI-TPα is involved in netrin-1-induced neurite outgrowth. Netrins are a family of secreted molecules that promote axon outgrowth by interacting with DCC (deleted in colorectal cancer) receptors. Since the mechanism of intracellular signalling of DCC was not yet clarified, the authors used the yeast-to-hybrid system to find interaction partners of DCC. PI-TPα was found as one of the interaction partners of DCC as well as neogenin (a netrin receptor in vertebrates [97]). The intracellular domains of the DCC receptor and neogenin bind to the C-terminal 20 amino acids of PI-TPα. This interaction seemed to be specific as no binding was found with PI-TPβ. The level of DCC-associated PI-TPα was increased when the neurons were stimulated with netrin-1, indicating that PI-TPα could be involved in netrin-1 signalling.

Lysates of cortical neurons that were incubated with netrin-1 showed an increased *in vitro* transfer activity of PI and PC. Similar results were obtained when neurons were pre-incubated with BDNF (brain-derived neurotrophic factor), another conserved signalling pathway of netrin-1 [98], but not with slit-2, a negative regulator of neurite outgrowth [99] or with lysates that were depleted of PI-TPα. In mice carrying a homozygous deletion of the intracellular domain in DCC, netrin-1-induced PI-TPα transfer activity was impaired. These results indicate that netrin-1 could increase PI-TPα transfer activity.

Phospholipid-binding assays showed that phosphoinositol-5-phosphate (PI(5)P) binds to the five C-terminal amino acids of PI-TPα, the same amino acids that are suggested to be responsible for the transfer activity. The authors suggested that PI(5)P plays a role in regulating PI-transfer activity and that binding to the DCC receptor may regulate binding of phospholipids to PI-TPα.

As PLC signalling is involved in neurite outgrowth [100] and since PI-TPα is suggested to be essential for PLC-regulated PIP_2 hydrolysis [66], it was investigated whether PI-TPα plays a role in outgrowth. Using neurons from the vibrator mouse, comparing neurons from wild type ($vb^{+/+}$), homozygous ($vb^{-/-}$) and heterozygous ($vb^{+/-}$) mice embryos, it was found that neurite outgrowth induced by netrin-1 was decreased in $vb^{-/-}$ neurons. However, netrin-1-independent outgrowth in all cultures was comparable. Similar results were obtained with BDNF.

To further investigate the involvement of PI-TPα in neurogenesis, knock-down of the protein was obtained in zebra fish embryos using antisense morpholino oligonucleotides (MO). At an intermediate dose of PI-TPα-MO, loss of spinal cord neurons was observed and, when present, axon outgrowth of remaining neurons was impaired.

In summary, the authors found that via DCC/neogenin, netrin-1 increases PI-TPα transfer activity. This elevates PIP_2 hydrolysis and stimulates netrin-1-induced neurite outgrowth. The exact mechanism by which PI-TPα transfer activity is increased remains unclear but the authors suggest a role for PI(5)P in the regulation of PI-TPα transfer activity.

4.5. PI-TPs and overexpression

Besides the analysis of the effect of PI-TP downregulation, cell lines have been generated in which PI-TPα or β were overexpressed. Wild type NIH3T3 mouse fibroblasts were stably transfected with DNA encoding for either PI-TPα or PI-TPβ generating SPIα (hence, sense PI-TPα) or SPIβ cells respectively.

4.5.1 SPIβ cells

In SPIβ cells PI-TPβ protein expression was shown to be upregulated 10-15 times [101]. Due to this high expression, the growth rate decreases compared to wild type fibroblasts. In addition, cell density was decreased upon confluency. Furthermore, and possibly correlated to the slow proliferation rate, the sensitivity of the SPIβ cells towards UV- and TNFα-induced apoptosis was enhanced. This increase in sensitivity seems to be dependent on the association of PI-TPβ with Golgi membranes since cells expressing the point mutant PI-TPβ^{S262A}, which does not associate with the Golgi membranes, show no change in apoptosis sensitivity compared to wild type fibroblasts [73]. Upon induced degradation of SM by a sphingomyelinase at the outside of the plasma membrane in SPIβ cells, it was shown that the SM level in the plasma membrane was restored more rapidly in these cells when compared with wild type fibroblasts. This indicates that PI-TPβ may be involved in maintaining the steady-state level of SM in the plasma membrane of SPIβ cells.

4.5.2 SPIα cells

In many aspects the phenotype and behaviour of SPIα cells mirrors the phenotype of SPIβ cells. SPIα cells show a 2-3-fold overexpression of protein PI-TPα. As a result, the doubling time of SPIα cells is decreased. In addition, the cell density upon confluency is increased [102]. An increased cell proliferation is often associated with an increase in resistance against apoptosis. Van Tiel and co-workers indeed showed that, in contrast to SPIβ cells, SPIα cells are extremely resistant towards UV- and TNFα-induced apoptosis [73, 103].

Analysis of phosphatidylinositol metabolites in SPIα cells showed an increased level of glycerophophoinositol, inositol-1-phosphate and inositol-2-phosphate and lyso-phosphatidylinositol (lyso-PI). Levels of PI and PIP_2 were found to be unchanged compared to wild type fibroblasts. The increased production of lyso-PI suggests that increased expression of PI-TPα activates a phoshpolipase A (PLA) [102]. The authors suggest that if a PLA_2 is activated, arachidonic acid (which is enriched in PI on the '*sn*-2' position [104]), may be released. Arachidonic acid is the precursor of eicosanoids, compounds that are known to play a role in many processes including cell proliferation [105], inflammation [106], and cell survival [107].

As it is known that eicosanoids are involved in mitogenesis, these compounds may act on SPIα cells in an autocrine fashion, thereby increasing their proliferation rate. Especially the cyclooxygenase-2 (COX-2) mediated arachidonic acid metabolites are known to play key roles in the above mentioned processes.

Schenning and co-workers [103] recently showed that in SPIα cells COX-2 expression was increased compared to wild type fibroblasts. By collecting medium in which SPIα cells were grown, it was shown that this conditioned medium (CM) exhibited mitotic and anti-apoptotic activity when incubated with wild type fibroblasts or SPIβ cells, indicating that factors responsible for this activity were secreted by SPIα cells. The activity of the CM was reduced when it was prepared in the presence of specific COX-2 inhibitors, suggesting that the secreted factors may be COX-2 derived arachidonic acid metabolites. In addition, the anti-apoptotic activity of the CM was partially blocked by inhibitors of G-protein coupled receptors (GPCRs) and an antagonist of the cannabinoid-1 receptor, a member of the GPCR family, indicating that a PI-TPα-dependent eicosanoid (i.e. arachidonic acid metabolite) or endocannabinoid may be involved.

From the data obtained with SPIα cells it can be concluded that, in contrast with degeneration that is associated with a reduction in the expression level of PI-TPα, increased expression of this protein is beneficial to fibroblasts in respect to protection against apoptosis. In addition, PI-TPα expression triggers other compounds in the cell, which results in the production and secretion of anti-apoptotic factors, most probably eicosanoids, which are able to protect other cells from apoptosis.

4.6. Function of PI-TPα

To date, the information derived from previous and above described data suggests a complex action for PI-TPα. A supposed function related to its lipid-binding and transport activity cannot be solely explained on this basis, as these features have only been described *in vitro*. A number of specific functions have been suggested for PI-TPα *in vivo*.

In permeabilised neuroendocrine cells, PI-TPα has been implicated as a soluble factor required for both secretory vesicle formation [108] and ATP-dependent priming of calcium-activated secretion [109]. In addition, PI-TPα has been shown to reconstitute GTP-γ-S-mediated PLC activity in permeabilised HL-60 cells, allowing the authors to suggest that PI-TPα is involved in transporting PI from intracellular compartments for conversion to PI(4,5)bisphosphate (PIP_2) [110]. Similar findings have been reported for epidermal growth factor-mediated stimulation of PLCγ and PI-4-kinase in A410 cells [111] and PLC-mediated PI degradation in carbachol-stimulated 1321N1 astrocytoma cells [112]. Further evidence suggests that PI-TPα increases inositol phosphate production by promoting the synthesis of PIP_2 [113, 114]. In cell-free mammalian models, PI-TPα has been shown to influence vesicle formation from the trans-Golgi network [115, 116].

However, up to now, genetic studies have failed to support these findings. Neither overexpression [102], nor downregulation [92, 117], nor knock-out of PI-TPα [95] in cells resulted in any alteration in phosphoinositide cycling. In addition, in knock-out stem cells, protein trafficking, endocytosis, biogenesis of secretory granules, and fusion of secretory granules with the plasma membrane appeared unchanged [95].

In addition, it was found that in the above described semi-*in vivo* systems (i.e. permeabilised cell system) no discrimination could be made between the function of PI-TPα and β. In view of the differences between the two proteins in e.g. cellular localisation and phenotype when overexpressed, the conclusions drawn above concerning the function of PI-TPα, may have to be re-evaluated.

5. Outline of this thesis

Despite intensive research, the function(s) of PI-TPα are as yet unclear. However, it is known that overexpression of PI-TPα in cells causes the production and secretion of one ore more anti-apoptotic factors. Interestingly, studies in fly and mouse revealed that a decrease in PI-TPα expression causes severe and progressive neurodegeneration. Together with the observation that PI-TPα expression is highest in CNS tissue, these findings imply that PI-TPα expression, and consequently its function, is essential in CNS maintenance.
In the remaining chapters of this thesis, this hypothesis will be elaborated and investigated in more detail using cellular models of neurodegeneration.
To analyse the relationship between PI-TPα expression and neurodegeneration we tried to answer the following questions:

- Does the conditioned medium of cells overexpressing PI-TPα exhibit neuroprotective properties?
- Which model systems can be developed to investigate the relationship between PI-TPα and neurodegeneration?
- Does the brain contain cells that resemble SPIα cells in producing and secreting PI-TPα-dependent survival-enhancing factors that are required for brain maintenance?

In ***chapter 2*** the neuroprotective property of the conditioned medium of SPIα cells (CMα) is analysed. The obtained data showed that CMα is able to protect primary-derived motor neurons against serum deprivation-induced cell death. The pathway by which this protection is accomplished leads most likely via a G-protein coupled receptor, as an inhibitor of this receptor family blocks the CMα-mediated protection.

In order to develop an *in vitro* model to investigate the relationship between PI-TPα and neurodegeneration in the CNS, PI-TPα expression in different CNS-derived cell lines and isolated primary cell cultures is analysed in ***chapter 3***. Like in SPIα cells, PI-TPα expression was found to be relatively high in astrocytes. Expression was significantly lower in neuronal cells. ***Chapter 4*** shows that CM of rat primary astrocytes as well as CM of the C6 astroglioma cell line is protective towards to the apoptotic-sensitive SPIβ cells. To investigate whether PI-TPα is responsible for the protective effect of this CM, in ***chapter 5***, PI-TPα protein expression was reduced in C6 astroglioma cells using RNA interference techniques. It was shown that the resulting CM demonstrates a decreased protective activity towards the motor neuron cell line NSC-34. This result indicates that the level of PI-TPα expression in astrocytes may be an important mediator of the secretion of neuroprotective factors.
Finally, in ***chapter 6***, all studies described in the former chapters are discussed and put into a general concept leading to a proposed model of PI-TPα in CNS maintenance.

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Chapter 2:

A PI-TPα-dependent survival factor protects cultured primary neurons against serum deprivation-induced cell death

Based on:

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Abstract

Selective neuronal loss is a prominent feature in both acute and chronic neurological disorders. Recently, a link between neurodegeneration and a deficiency in the lipid transport protein phosphatidylinositol transfer protein α (PI-TPα) has been demonstrated. In this context it may be of importance that fibroblasts overexpressing PI-TPα are known to produce and secrete bioactive survival factors that protect fibroblasts against UV-induced apoptosis. In the present study it was investigated whether the conditioned medium of cells overexpressing PI-TPα (CMα) has neuroprotective effects on primary neurons in culture. We show that CMα is capable of protecting primary, spinal cord-derived motor neurons from serum deprivation-induced cell death. Since the conditioned medium of wild type cells was much less effective we infer that the neuroprotective effect of CMα is linked (in part) to the PI-TPα-dependent production of arachidonic acid metabolites. The neuroprotective activity of CMα is partly inhibited by suramin, a broad-spectrum antagonist of G-protein coupled receptors. Western blot analysis shows that brain cortex and spinal cord have relatively high levels of PI-TPα suggesting that the survival factor may be produced in neuronal tissue. We propose that the bioactive survival factor is implicated in neuronal survival. If so, PI-TPα could be a promising target to be evaluated in studies on the prevention and treatment of neurological disorders.

Introduction

Neurodegenerative disorders are marked by selective and progressive neuronal loss with consequent neurological deficits. Insight into the mechanisms controlling neuronal survival and death may provide important clues regarding the development of neuroprotective strategies. Interestingly, (motor) neuron degeneration has recently been linked to the reduced expression of phosphatidylinositol transfer protein α (PI-TPα). Thus, the so-called vibrator mouse, that suffers from progressive action tremor caused by degeneration of motor neurons in the cerebellum, brainstem and spinal cord, showed a reduction in brain PI-TPα expression [1]. Moreover, PI-TPα knock-out mice die within 14 days after birth due to progressive spinocerebellar degeneration [2]. On the other hand, by using a NIH3T3 mouse fibroblast cell line overexpressing PI-TPα, it has been shown that the overexpression of PI-TPα is coupled to enhanced cellular protection [3].

PI-TPα belongs to the family of lipid transport proteins, and is highly conserved throughout mammalian evolution. At least in vitro it has been shown that PI-TPα binds and transfers phosphatidylinositol and phosphatidylcholine between membranes [4]. In mice, the highest abundance of PI-TPα has been reported to occur in brain [5].

Analysis of the inositol metabolites in PI-TPα-overexpressing cells indicated the activation of a phospholipase A_2 (PLA_2) with affinity towards PI, resulting in an enhanced production of lysophosphatidylinositol and other degradation products like arachidonic acid and its metabolites [3, 6].

Interestingly, conditioned medium from PI-TPα-overexpressing cells (CMα) has been demonstrated to stimulate the rate of proliferation of wild type NIH3T3 cells and to confer protection against UV radiation and TNFα treatment. This indicates that PI-TPα-overexpressing cells secrete both PI-TPα-dependent mitogenic and survival factors. Since the production and metabolism of arachidonic acid is increased in the PI-TPα overexpressing fibroblasts, the protective property of CMα may be attributable, at least in part, to arachidonic acid derived compounds [3]. This is corroborated by the observations that (i) an arachidonic acid enriched lipid extract of CMα expresses a protective activity similar to that of CMα, and (ii)

inhibition of cyclooxygenase-2 (COX-2) reduces the anti-apoptotic efficacy of CMα.

Given the relationship between PI-TPα expression and neurodegeneration in transgenic mouse models, and the profound anti-apoptotic efficacy of CMα, we hypothesise that PI-TPα may be critically involved in the production of a survival factor that is implicated in neuronal survival. To test this hypothesis, we have investigated and characterised the effect of CMα on the survival of serum-deprived neurons, using spinal cord-derived rat motor neurons as a model.

Materials and methods

Chemicals

Trypsine (Calbiochem), laminine (Life Technologies), culture media and sera (Gibco), glycin (Baker), paraformaldehyde (Riedel de Hähn), goat-anti-rabbit horseradish peroxidase antibody (Biorad), mowiol 4-88 (Calbiochem) and enhanced chemilumincence detection reagents (Amersham Biosciences) were obtained from the suppliers indicated. SR141716A was a gift from dr. G. van Zadelhoff (Section Bio-organic Chemistry, Bijvoet Centre, Utrecht University). Unless mentioned otherwise, all other chemicals were obtained from Sigma.

Primary motor neuron culture

Motor neurons were isolated from E15 Wistar rat embryonic spinal cord by metrizamide density centrifugation as previously described [7, 8]. In brief, the ventral part of the spinal cord of 3 embryos was dissected and cut into small pieces, before being trypsinised (0.05% w/v) for 15 min at 37°C. After degrading liberated DNA with 100 μg/ml DNAse I, the cell suspension was put on a 6.5% metrizamide cushion and centrifuged for 15 min at 790g. Cells from the interphase were collected, put on a 4% BSA cushion, and centrifuged at 170g for 10 min. Finally, the pellet was resuspended in motor neuron culture medium, and plated in polyornithine (1.5 μg/ml) and laminine (3 μg/ml) coated 24 wells plates at a density of 12,000 cells per well. Cells were grown at 37°C, 6% O_2, and 5% CO_2 in a humidified atmosphere.

Motor neuron culture medium (MN medium) consisted of L15 medium containing $NaHCO_3$ (22 mM), insulin (5 μg/ml), putrescine (0.1 mM), conalbumin (0.1 mg/ml), sodium selenite (30 nM), glucose (20 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and embryonic chicken muscle extract (ECE, 1.2% [9]).

Primary astrocyte culture

To obtain primary astrocyte cultures, the pellet fraction obtained after the metrizamide step in the motor neuron purification procedure was resuspended in culture medium. Cells were plated in polyornithine (1.5 μg/ml)-coated 75 cm^2 culture flasks and grown to confluence at 37°C, and 5% CO_2. Subsequently, non-astrocytic contaminating cells were shaken off on a rotary shaker (250 rpm; over night; 37°C) [10]. Culture medium was L15 medium containing $NaHCO_3$ (22 mM), glucose (20 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and FBS (10%).

Preparation of conditioned media (CM) and lipid extract (LE)

CM was prepared as described previously [3]. Briefly, cell cultures of either wild type NIH3T3 mouse fibroblasts or NIH3T3 cells overexpressing PI-TPα (SPIα cells) were grown to 80-90% confluence in DMEM containing 10% newborn calf serum before the medium was replaced by ECE-free MN medium containing 0.1% BSA. After 24 h, the medium was collected and centrifuged (5 min at 1,000 rpm) to yield either CMα or CMwt (from SPIα or wild type NIH3T3 cells, respectively). Conditioned media were stored at 4°C until further use.

Lipid extracts (LEs), containing arachidonic acid metabolites, were obtained from CMα and CMwt as described previously [11]. In short, upon addition of 0.03 ml of 12 M formic acid to one ml of CM the mixture was extracted with two 3 ml volumes of ethyl acetate. The combined extracts were then evaporated under nitrogen before the residues were dissolved in ethanol to yield LEα and LEwt, respectively, which were immediately used after preparation.

Experimental design

The effect of CM and LE on the survival of motor neurons upon serum deprivation was monitored as a function of time. Therefore a healthy

subpopulation of motor neurons (± 25 neurons/well) was identified 4 days after plating by phase contrast microscopy and mapped with a Leica DM IRBE microscope (Rijswijk, The Netherlands) equipped with the Leica Quantimet images analysis system software. Healthy motor neurons were defined as phase bright cells containing neurites longer than 2 cell diameters [7, 8, 12]. Serum deprivation-induced cell death was induced by replacing the medium with ECE-free MN medium. The effect of CMα, CMwt, and the respective LEs on survival was assessed by supplementing the ECE-free MN medium with various dilutions of these media. When motor neurons growing on 1 cm^2 of well surface were incubated with CM from 1 cm^2 of confluent fibroblasts, this condition was defined as incubation with undiluted CM (1:1). Accordingly, a 3 times dilution of CM (CM 1:3) means that motor neurons growing on 3 cm^2 of surface of were incubated with CM from 1 cm^2 of fibroblasts.

At different time points following the start of serum deprivation, the viability of mapped motor neurons was assessed under phase contrast microscopy using well-established criteria to distinguish between healthy and dead cells (intact cell body and processes vs. fragmented processes and the presence of granule- and vacuole-like structures in the cell body, respectively) [13].

When indicated, the effect of the broad-spectrum antagonist of the G-protein coupled receptor family suramin (100 μM) [14], or the cannabinoid 1 receptor antagonist SR141716A (rimonabant; 10 μM) [15] was tested on the CMα-induced neuroprotection as observed within the first 24 h of treatment.

Immunocytochemistry

Primary motor neurons and confluent astrocyte cultures, grown on polyornithine and laminine- or polyornithine-coated glass cover slips, respectively, were washed with PBS (pH 7.4), and fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. After washing with PBS, the cells were permeabilised in 0.5% Triton X-100 in PBS for 5 min and, after an additional washing step, incubated for 10 min in 50 mM glycine in PBS. Blocking occurred in HEPES-buffered DMEM containing 0.5% BSA for 30 min.

PI-TPα immunoreactivity was demonstrated using an anti-PI-TPα polyclonal antibody (1:100) [16]. After 60 min, the cells were washed and incubated with goat-anti-rabbit-Cy3 for 60 min. Antibodies were diluted in DMEM-HEPES containing 0.1% BSA. Cells were mounted in mowiol 4-88 containing 0.1% 1,4-

diazabicyclo[2.2.2.]octane. Fluorescence was visualised by confocal laser scanning microscopy (CLSM), using a Nikon Eclipse TE2000-U microscope, equipped with a confocal C1 unit. Cy3 was excited with the 543 nm line of a He-Ne laser and a 585/30 emission filter.

Analysis of PI-TPα expression in mouse CNS tissue

Three female C57BL/6J@Rj mice (Jackson laboratories, Bay Harbour, ME, USA), 90 days of age, were anaesthetised and decapitated. Quickly, the liver, cortex and the whole lumbar part of the spinal cord were collected in ice cold lysis buffer (50 mM Tris-HCl pH 7.4, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM $NaVO_4$; 20 μl/mg tissue). The tissue was homogenised, centrifuged at 10,000 g for 10 min before the supernatant was stored at -20°C until use. Aliquots of 20 μg of protein were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. 10, 25 and 50 ng of purified PI-TPα was used as a calibration curve. After blocking in non-fat milk powder for 1 h, membranes were incubated with an affinity-purified polyclonal rabbit antibody raised against synthetic peptides representing specific epitopes of PI-TPα (1:500) [16] for 1 h. After washing, a goat-anti-rabbit antibody conjugated to horseradish peroxidase (1:5000) was used as a secondary antibody (1 h). Membranes were washed and developed with enhanced chemilumincence detection reagents according to the manufacturer's instructions.

Statistical analysis

For the multiple comparisons between groups, after testing for the homogeneity of variance and for normality of residuals, an analysis of variance (ANOVA) was performed. p-values <0.05 were considered statistically significant.

Results

The effect of CMα on serum deprivation-induced motor neuron death

Serum deprivation is a well-known inducer of cell death in neuronal cell lines as well as in primary neuronal cultures [17]. As illustrated in Figure 2.1, discrimination between viable and dead motor neurons was made on a morphological basis. While viable motor neurons were defined as cells containing intact cell bodies and processes (Panel A), degenerating, dead cells were distinguished by the presence of fragmented processes and granule- and vacuole-like structures within the cell body (Panel B). It has previously been demonstrated that the discrimination between healthy and dead cells on the basis of phase contrast microscopy strongly correlates with live/death assays. [18]. Table 2.1 shows that incubation of primary motor neurons with ECE-free MN medium resulted in a marked cell loss of approximately 70 % within 96 h. However, when motor neurons were grown in

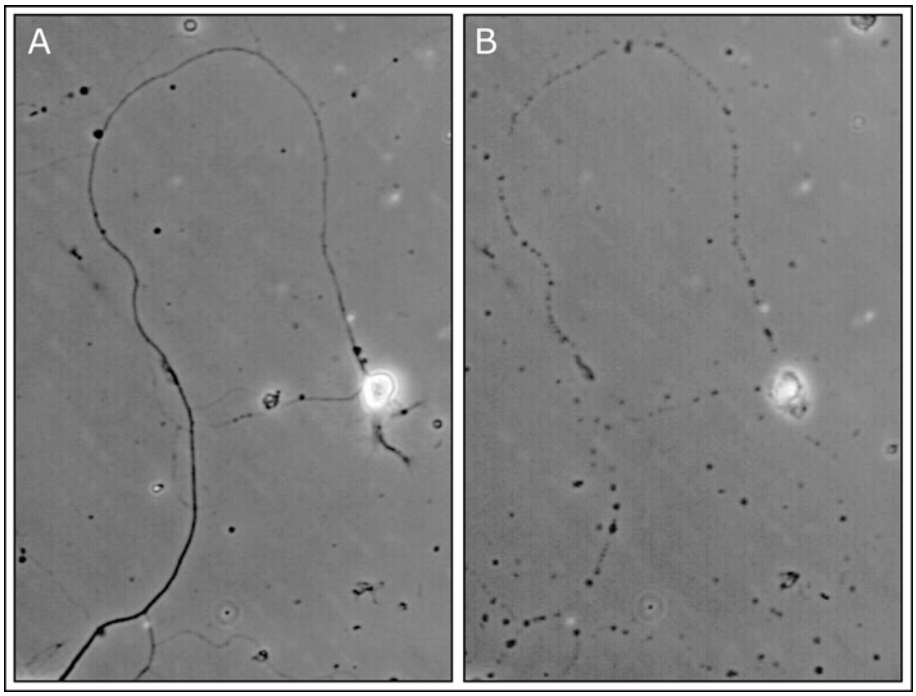

Figure 2.1: Phase contrast microscope images showing primary motor neurons in culture. The images show the same neuron before (Panel **A**) and after (Panel **B**) 24 h of serum deprivation. Note fragmented processes and the presence of granule- and vacuole-like structures in the cell body of the degenerating motor neuron in panel B.

Table 2.1 The neuroprotective effect of CMα on primary motor neurons.

Condition	Survival at 96 h (as % of t = 0 h)
ECE-free MN medium	31 ± 4
+ CMα 1:100	41 ± 11
+ CMα 1:30	45 ± 12
+ CMα 1:10	67 ± 4 *
+ CMα 1:3	80 ± 3 *
+ CMα 1:1	68 ± 9 *

the presence of CMα the survival was greatly increased. As shown, a concentration-dependent neuroprotective effect was observed, with a 1:3 dilution of CMα showing optimal protection. Under this condition, cell survival increased from 31 ± 4 to 80 ± 3% (mean ± SEM) at 96 h. When tested at higher concentrations (i.e. up to 1:1), CMα appeared to be less effective which may be explained by the presence of some toxic factors in the same medium.

The effect of CMwt on serum deprivation-induced motor neuron death

To investigate whether the overexpression of PI-TPα in SPIα cells is indeed responsible for the neuroprotective activity of CMα, also the effect of conditioned medium of wild type NIH3T3 fibroblasts (CMwt) was tested. As shown in Figure 2.2, during the first 24 h of treatment, motor neurons treated with CMwt at a dilution of 1:3 also showed increased survival when compared to control cells (94 ± 3 vs. 58 ± 8%, respectively). After 24 h however, motor neuron loss in the CMwt treated wells proceeded in a similar way to that observed in the control cells treated with ECE-free MN medium. Thus, the percentage of cell loss in cultures treated with CMwt amounted to 48 ± 4% between 24 and 96 h, which was not statistically different from that observed in control cells (54 ± 7%). At all time points studied,

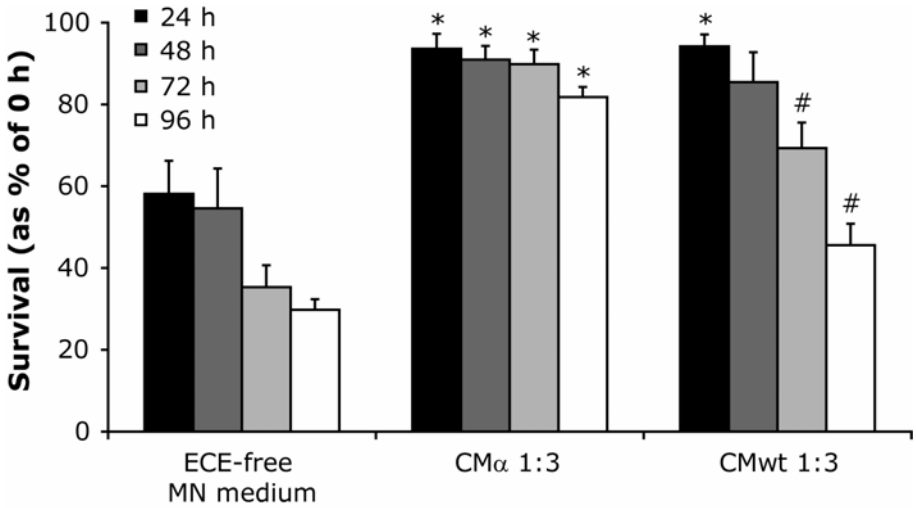

Figure 2.2: CMα, but not CMwt, protects primary motor neurons against serum deprivation-induced cell death. Four days after plating, motor neurons were incubated in ECE-free motor neuron medium, CMα at the optimal concentration (1:3) or the same concentration of CMwt. Survival was scored after 24, 48, 72 and 96 hours under phase contrast microscopy using well defined morphological criteria and expressed as percentage of the survival at the start of the treatment. Data are mean ± SEM of 8 observations determined in 2 independent experiments. $^{*}p < 0.05$ relative to the survival in ECE-free MN medium at the same time point. $^{\#}p < 0.05$ the survival observed in CMwt-treated cultures vs. CMα-treated cultures at the same time point.

the 1:3 dilution of CMα was more effective in enhancing the survival of motor neurons as compared to CMwt.

As wild type NIH3T3 fibroblasts do express some endogenous PI-TPα [6], CMwt was also tested at higher concentrations, since the amount of survival factor may be limited in CMwt. However, even at a three times higher concentration, or after renewed addition of CMwt (1:3) after 48 h of treatment, CMwt was not able to prevent motor neuron death (data not shown). Taken together, these data indicate that overexpression of PI-TPα is required for the production and secretion of the survival factors involved in preventing motor neuron death.

The effect of a lipid extract of CMα on serum deprivation-induced motor neuron death

Since the production and metabolism of arachidonic acid is increased in SPIα cells [3], it was tested whether a lipid extract derived from CMα (LEα), containing arachidonic acid metabolites, could protect primary motor neurons. As illustrated in Figure 2.3, LEα protected motor neurons almost completely up to a period of 72 h whereas LE obtained from CMwt (LEwt) was virtually inactive. On the basis of the amount of CMα extracted, LEα was approximately 30 times less efficient than CMα in providing optimal protection. This may indicate either that the lipid factors including the arachidonic acid metabolites in CMα are only partially responsible for the protective effect or that the active metabolites are rapidly inactivated upon extraction.

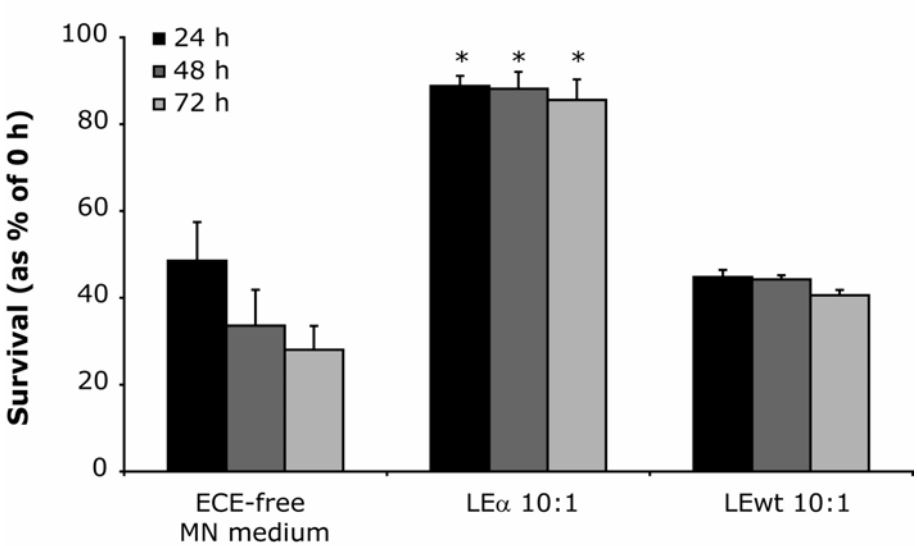

Figure 2.3: Survival of primary motor neurons incubated with a lipid extract obtained from CMα and CMwt, containing arachidonic acid metabolites (LEα and LEwt, respectively). Incubation with ECE-free MN medium served as a control. Survival was scored 24, 48 and 72 hours after treatment and expressed as percentage of the survival at the start of the treatment. Data are mean ± SEM from 12 observations determined in three independent experiments. $^{*}p < 0.05$ relative to the survival in ECE-free MN medium at the same time point.

The effect of suramin and rimonabant on CMα-induced neuroprotection

In a previous study evidence was obtained that the survival factor in CMα may act on a cannabinoid 1-like receptor (CB1R) a member of the family of G-protein

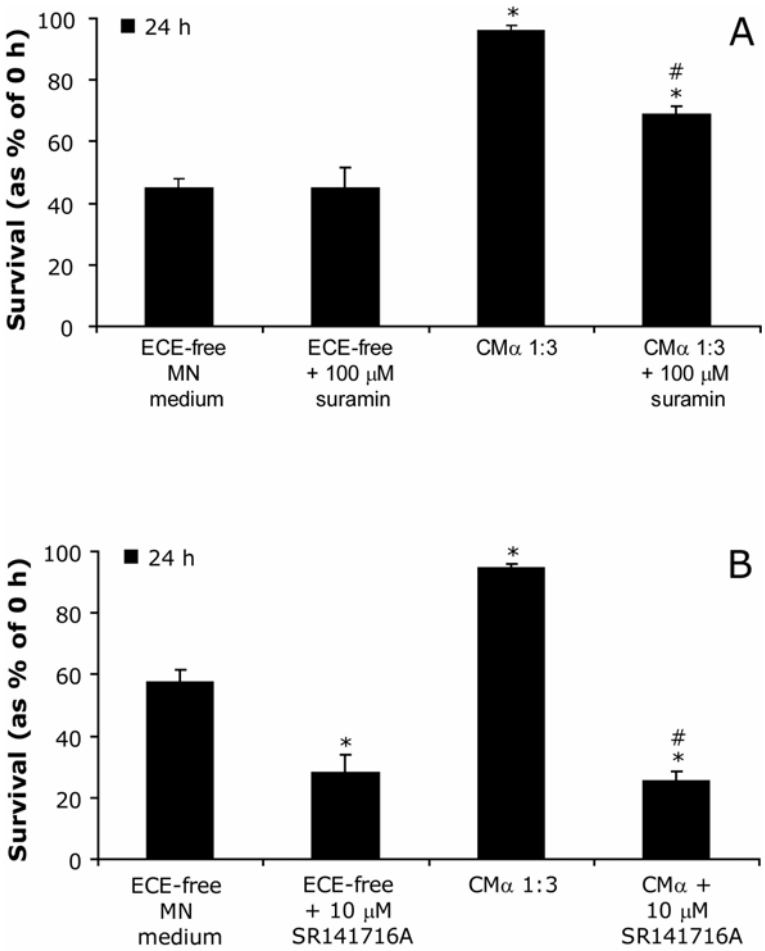

Figure 2.4: The role of G-protein coupled receptors in the CMα-mediated effect. Four days after plating, primary motor neurons were treated with the optimal concentration of CMα with or without (Panel A) 100 μM suramin, a broad-spectrum antagonist of G-protein coupled receptors, or (Panel B) 10 μM SR141716A (rimonabant), a specific antagonist of the cannabinoid 1 receptor. ECE-free MN medium with or without these compounds served as a control. Survival was scored after 24 h. Data are mean ± SEM from 8 observations obtained in two independent experiments. $^{*}p < 0.05$ relative to the survival in ECE-free MN medium. $^{\#}p < 0.05$ vs. the survival observed in CMα-treated cultures.

coupled receptors (GPCR) [3, 19].

To further characterise the neuroprotective effect of CMα in our neuronal model, the effect of CMα was tested in the presence of suramin, a broad-spectrum non-specific antagonist of GPCRs, and SR141716A (rimonabant, acomplia™), a specific antagonist of the CB1R (Figure 2.3). While suramin (100 μM) itself had no effect on the survival of motor neurons in ECE-free MN medium, co-incubation with this antagonist resulted in a 50% decrease of the neuroprotective effect of CMα (Panel A). Similarly, in the presence of 10 μM rimonabant, a 75% decrease in the CMα-mediated neuroprotective effect could be observed (Panel B). However, at the concentration used, this particular antagonist already caused a 50% decrease in motor neuron viability in the control situation.

PI-TPα expression in cultured (non-)neuronal cells and mouse CNS

Previously, it has been demonstrated that PI-TPα is most abundant in total brain homogenate [5]. In agreement with this, we found that PI-TPα levels in mouse brain cortex (CTX) but also in lumbar spinal cord (LSC) tissues are much higher than the levels detected in the liver (LIV) (Figure 2.5). By using a calibration curve based on purified PI-TPα, and with the conformation of equal protein loading by means of Coomassie Brilliant Blue staining of a parallel-runned gel, it could be estimated that PI-TPα levels in cortex are about twice as high as compared to lumbar spinal cord.

By using a polyclonal antibody the cellular expression of PI-TPα was investigated in primary spinal cord-derived motor neurons (grown for four days in MN

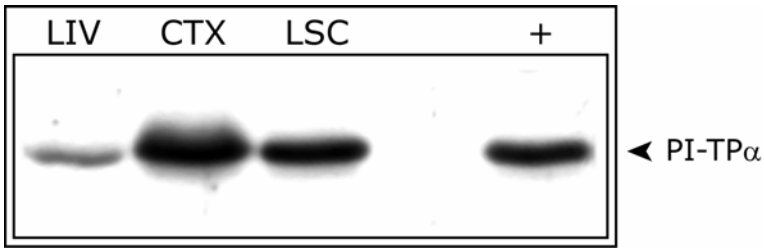

Figure 2.5: Expression of PI-TPα in tissue. Protein homogenates (20 μg) from female C57BL/6J@Rj mouse liver (LIV), cortex (CTX) and lumbar spinal cord (LSC) tissue were resolved by SDS-PAGE and analysed by Western blot analysis. While Coomassie Brilliant Blue staining was exploited to confirm equal loading, 10, 25 and 50 ng of purified PI-TPα was used as a calibration curve.

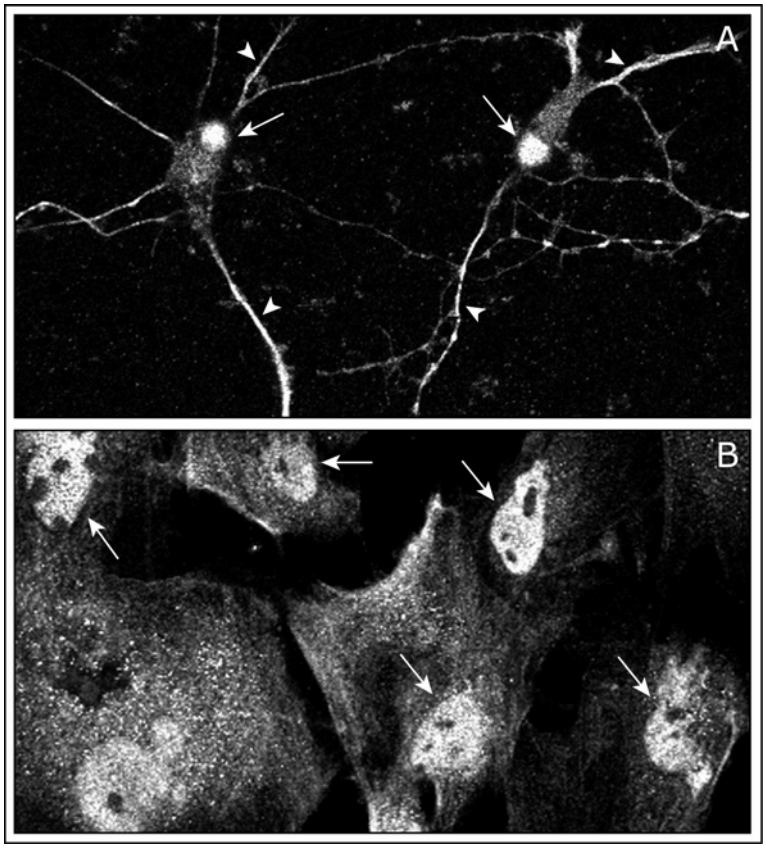

Figure 2.6: CLSM images showing the cellular expression of PI-TPα in primary motor neurons (Panel **A**) and embryonic spinal cord astrocytes (Panel **B**). PI-TPα is predominantly expressed in the nuclei of both cell types (arrows) with moderate expression being observed in the cytosol. Arrowheads indicate the marked staining of PI-TPα in the dendritic and axonal processes of the motor neurons.

medium) and primary astrocyte cultures. As shown in Figure 2.6, PI-TPα is markedly expressed in the nuclei of both motor neurons (Panel A) and astrocytes (Panel B). Moreover, besides a moderate expression in the cytosol of both cell types, marked staining was observed in the dendritic and axonal processes of the motor neurons. Overall, this cellular expression pattern is comparable with that previously observed in NIH3T3 fibroblasts [20].

Discussion

In this study we report that the medium conditioned by mouse fibroblasts overexpressing PI-TPα protects primary motor neurons against serum deprivation-induced cell death. Present and previous data indicate that COX-2-derived lipid factors (including endocannabinoids) secreted into the conditioned medium are partially responsible for this effect [3]. Moreover, we found that part of the protective effect was inhibited by a broad-spectrum antagonist of GPCRs, indicating that this type of receptor is involved in the protective mechanism.

A direct link between PI-TPα and neurodegeneration became first apparent from the observation that mice homogenous for the so-called vibrator mutation develop action tremor, brainstem and spinal cord degeneration and, as a consequence, juvenile death [1]. Embryonic development, however, is normal in these animals. In the vibrator mouse, PI-TPα mRNA as well as protein levels were found to be 80% reduced due to an insertion of a transposable element into intron 4 of the PI-TPα gene. The level of PI-TPβ, an isoform of PI-TPα, was not altered. The authors suggested that a defect in a PI-TPα-dependent PI-turnover pathway may be implicated in specific neurodegenerative diseases.

In another study, deletion of PI-TPα was established by ablation of the gene in mice [2]. Similar to vibrator mice, PI-TPα appeared not to be required for prenatal development of the PI-TPα$^{-/-}$ mice. However, after birth PI-TPα$^{-/-}$ mice showed a lower body mass, hypoglycaemia, and spinocerebellar degeneration characterised by reactive gliosis of the cerebellum and brain stem and white and grey matter deficits in the spinal cord. Reportedly, PI-TPα$^{-/-}$ mice do not survive beyond 14 days because of ongoing neurodegeneration. It can be concluded from these studies that, although expression of PI-TPα is not essential for normal embryonic brain development (probably due to in utero supply of maternal nutrients), this protein is necessary for normal development and maintenance of neural tissues in newborn mice.

Previously, it was shown that NIH3T3 fibroblasts overexpressing PI-TPα (SPIα cells) exhibited an increased survival upon induction of apoptosis in vitro [6]. Moreover, medium conditioned by SPIα cells protected wild type cells from UV- and TNFα-induced apoptosis. It was shown that the overexpression of PI-TPα stimulated the activation of a PLA_2 with affinity towards PI, leading to the release

of arachidonic acid. Since COX-2 inhibition reduced the production of the PI-TPα-dependent survival factors it was concluded that arachidonic acid is the actual precursor of the survival factor [3].

Although PI-TPα is ubiquitously expressed in mammalian tissues, it is to be noted that highest levels are found in brain [5]. As demonstrated in the present study, PI-TPα is expressed in both cortex as well as spinal cord at levels significantly higher than in liver (Figure 2.5). Moreover, we have shown by immunocytochemistry that PI-TPα can be detected in cultured primary motor neurons as well as in astrocytes (Figure 2.6). On the basis of these observations it is tempting to hypothesise that a

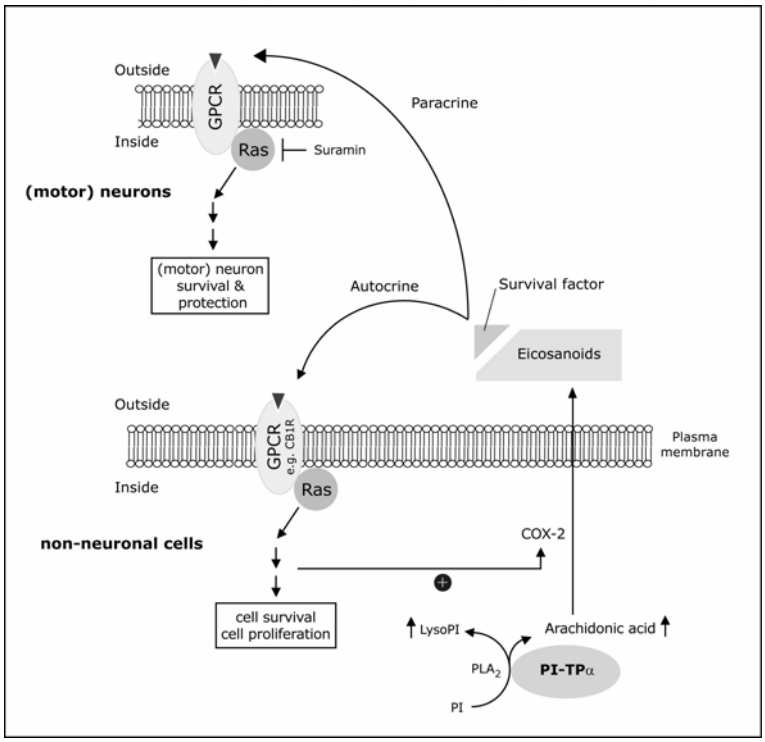

Figure 2.7: Hypothetical regulatory role of PI-TPα in the production of a neuroprotective eicosanoid in the CNS. High expression of PI-TPα results in activation of a phospholipase A_2 (PLA_2) with affinity towards Phosphatidylinositol (PI) and, as a consequence, increased production of arachidonic acid and lysophosphatidylinositol. Arachidonic acid is converted by COX-2 to yield eicosanoids including the survival factor. In turn, by stimulating GPCRs, this factor is proposed to enhance cell survival and to confer neuroprotection in both an autocrine and a paracrine manner. In addition, the survival factor-mediated activation of GPCRs may result in an upregulation in COX-2 expression, thus creating a positive feedback loop.

similar PI-TPα-driven production of a neuroprotective factor is operative in brain tissue. This hypothesis is supported by the following data.

First, besides PI-TPα, also the other two proteins critically involved in the PI-TPα-dependent generation of the survival factor(s), (i.e. COX-2 and PLA_2) are known to be expressed in brain under normal conditions [21, 22]. Moreover, while astroglial and neuronal COX-2 was found to be upregulated in Parkinson's and Alzheimer's diseased brains [23-25], PLA_2 and COX-2 mRNA and protein levels are markedly upregulated in early-stage amyotrophic lateral sclerosis (ALS) and ALS transgenic mice models [24, 26, 27]. Not to deny the 'dual role' of COX-2 in the pathophysiology of neurodegenerative diseases [28], at least in respect to the production of the PI-TPα-dependent survival factor COX-2 expression is hypothesized to constitute a beneficial rather than a detrimental feature.

Secondly, there is accumulating evidence for the emerging role of the endocannabinoid system and endocannabinoid signalling in the maintenance of neuronal integrity and function [29]. From previous studies using mouse fibroblasts, evidence was obtained that (one of) the PI-TPα-dependent survival factor(s) is (are) a COX-2-dependent endocannabinoid [3]. As reviewed by [30] endocannabinoids are known to protect against acute brain damage most likely by acting on the cannabinoid (-like) receptors which are widely distributed in the CNS [31]. In addition, the neuroprotective effect of cannabinoids has also been reported in models of Huntington's disease, ALS, Parkinson's-, and Alzheimer's disease [32-36]. In our study the broad-spectrum GPCR antagonist suramin partially inhibited the neuroprotective activity of CMα on motor neurons, indicating that GPCR activation may be similarly involved in the neuroprotective action of CMα on these cells. However, the identification of the specific GPCR involved, and the putative role of CB1R in the neuroprotective effect of CMα, remains to be elucidated. Although the CB1R antagonist rimonabant strongly attenuated the CMα-induced neuroprotective effect, the antagonist itself resulted in a neurotoxic effect in control neurons, indicating that primary motor neurons depend on CB1R activation. This supports our hypothesis that the survival factors in CMα may display agonistic activity on CB1(-like)Rs and thereby regulate neuronal protection and maintenance.

Finally, using mouse fibroblasts it was shown that the PI-TPα-dependent survival factor(s) are able to act by autocrine as well as paracrine pathways [3]. Although it cannot be excluded that the neuroprotective factors are produced elsewhere in the

body, we propose on the basis of our immunocytochemical and Western blot analysis, a paracrine protection pathway, in which astrocytes are likely candidates in providing the neuroprotective factor to neuronal cells. In this respect it should be emphasized that for optimal production and/or activation events, all proteins involved in the processes should be expressed at an appropriate level in particular cells.

Our data, together with the notion that all components required to produce the survival factor are present in brain, support our hypothesis that the PI-TPα-dependent survival factor may be implicated in the survival of (motor) neurons both in health and disease (see Figure 2.7). We conclude that PI-TPα is a promising target to be evaluated in studies to the prevention and treatment of neurodegenerative disorders.

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Chapter 3:

In vitro models to investigate the role of PI-TPα in neuroprotection and neurodegeneration

Hanneke Bunte, Gerry T. Snoek, Karel W.A. Wirtz and Freek L. van Muiswinkel

Abstract

Recently, we found that a PI-TPα-dependent survival factor protects cultured primary neurons against serum deprivation-induced cell death. In order to further investigate the relationship between PITPα and neurodegeneration, cell line-based models are developed that allow large-scale biochemical analyses and experimental manipulation of the PI-TPα expression. To this end, besides primary motor neuron and astrocyte cultures, a selection of cell lines, derived from the central nervous system, including C6 astroglioma and the motor neuron-like NSC-34 cell line were evaluated for the PI-TPα content. In addition, the latter two cell lines were evaluated for their use in a co-culture system as a model for the role of PITPα in neuron-glia interactions *in vivo*.

Introduction

To study the role of PI-TPα in neurodegeneration, currently two *in vivo* mouse models are available, notably the vibrator mouse with a spontaneous developed insertion in the *pitn* gene resulting in a 80% reduction of PI-TPα protein expression [1, 2] and the PI-TPα knock-out mouse [3]. So far, these models provided ample information about the effect of PI-TPα downregulation on the organism as a whole, with severe neurodegeneration of the motor system being one of the most striking phenomena in both models. The exact relationship between PI-TPα expression and neurodegeneration, however, has not been elucidated yet. Since the central nervous system (CNS) is such a complex organ, pathogenic mechanisms and detailed biochemical aspects of the CNS are difficult to investigate *in vivo*. Hence, notwithstanding the great value of the above animal models, studies on the pathophysiological role of PI-TPα in the CNS, including large-scale biochemical investigations, will require the development of suitable *in vitro* models.

Currently, some well-characterised cell lines are available to investigate the function of PI-TPα in general. Thus, in addition to embryonic stem cells in which PI-TPα was ablated [4], genetically-engineered NIH3T3 fibroblast cell lines overexpressing PI-TPα or PI-TPβ (SPIα and SPIβ cells, respectively) have been generated [5]. Interestingly, where SPIβ cells showed an increased sensitivity towards UV- or serum deprivation-induced apoptosis, the SPIα cells showed a highly reduced sensitivity and appeared to be less vulnerable to these types of insult [6, 7]. When it was found that SPIα cells may secrete PI-TPα-dependent survival-enhancing factors, one has set up an *in vitro* model to test the hypothesis that these factors may have a paracrine mode of action. To this end, in addition to the use of conditioned media from both SPIα and wild type NIH3T3 control cultures, various biochemical and pharmacological approaches have been used to try and characterise the biological origin and working mechanism of these factors [6, 7]. As depicted in Figure 3.3A, in this original NIH3T3/SPIα/SPIβ model SPIα cells served as survival factor-'producing cells', with NIH3T3 cells as control cells, while the apoptosis-sensitive SPIβ cells were used as 'target cell line' to assess the survival-enhancing activity of factors secreted by SPIα [7].

As far as the use of CNS-derived models is concerned, in 2005 Xie and co-workers reported the use of both primary neuronal and organotypic cultures derived from cortical tissue of vibrator mouse embryos to study the involvement of PI-TPα in netrin-1-induced neurite outgrowth [8].

Prompted by the above *in vivo* observations on the degeneration of the motor neuron system in PI-TPα deficient mice, we recently started to use primary motor neuron cultures and SPIα cells to investigate the possible relationship between PI-TPα expression and motor neuron survival. As reported in chapter 2, we have found that a PI-TPα-dependent survival factor is capable to protect cultured primary motor neurons against serum deprivation-induced cells death [9]. Thus, in line with the observation that downregulation of PI-TPα results in the loss of motor neurons *in vivo*, we discovered that conditioned medium derived from SPIα cells enhances the survival of motor neurons *in vitro*.

However, SPIα cells might not be the most obvious cell type to model cellular interactions in the CNS and the isolation of primary (neuronal and non-neuronal) cells is often a time-consuming procedure and requires the sacrifice of laboratory animals. Therefore, we have extended the investigation on the relationship between PI-TPα and neurodegeneration by searching for alternative cell models, preferably based on well-characterised cell lines rather than primary cultures. The advantage of such an approach is given by the fact that these models allow large-scale biochemical analyses and are generally more tolerant to the conditions required for experimental manipulation of the level of PI-TPα expression.

Although the NIH3T3/SPIα/SPIβ cell model has been very instrumental in interpreting some of the basic aspects of the biological function of PI-TPα, it is evident that these cell lines were not intended to serve as a model to investigate its putative role in neuronal survival. Since it was shown that the PI-TPα-dependent survival-enhancing factors, secreted by SPIα cells are able to protect SPIβ cells as well as primary motor neurons, and PI-TPα is abundantly expressed in the CNS [6], we hypothesised that a similar endogenous paracrine mode of action may be operational in the CNS. Moreover, given the important role of non-neuronal glial cells in neuronal development and maintenance, we speculated that non-neuronal cells, and astrocytes in particular, may be likely candidates to serve a role in the production of PI-TPα-dependent factors in CNS tissues. Thus, when developing *in vitro* models to elucidate the putative relationship between PI-TPα and

neurodegeneration, the role of glial cells in producing PI-TPα-dependent survival factors should also be considered. Hence, to substitute for the artificial SPIα cells, cultures of (primary) astroglial cells will be included in the present studies.
In respect to our ambition to ultimately develop cell line-based models, it is noteworthy that various immortalised cell lines, in which (several of) the original properties of primary cells are preserved, are currently available. In general, cell lines are easy to handle and constitute an unlimited self-replicating source that can be grown in almost infinite quantities. In addition, they exhibit a relatively high degree of homogeneity and can be easily replaced from frozen stocks. As far as the alternatives for primary motor neurons are concerned, over the past decades several motor neuron cell lines have been created. Of these, the well-characterised motor neuron-like cell line NSC-34, originally developed by Cashman and co-workers in 1992 [10], is commonly used. In the present study, this cell line, that comprises a hybrid between neuroblastoma cells and spinal cord-derived neurons, will be evaluated as 'target cell' in our attempt to set up a new cell-line based *in vitro* model to study the role of PI-TPα in neuron-glia interactions. Similarly, in the current study, the well-characterised and generally accepted C6 astroglioma cell line [11] will be evaluated as an alternative for primary astrocytes.

As a first step in the development of tailor-made *in vitro* models suitable to further our studies into the relationship between PI-TPα and neurodegeneration, in the present study, several of the above described neuronal as well as non-neuronal cell cultures will be analysed for their PI-TPα content. As revealed by previous studies using the NIH3T3/SPIα/SPIβ model, a relatively high level of PI-TPα is essential to produce and secrete the PI-TPα-dependent survival-enhancing factor(s) [7]. Hence, the level of PI-TPα expression in the different cell cultures will be determined by Western blot analysis using a well-characterised polyclonal rabbit antibody raised against synthetic peptides representing specific epitopes of PI-TPα [12]. Based on these data, a subset of primary and immortalised cells will be selected to serve either as 'producers' of the PI-TPα-dependent bio-active factor(s) or as 'target cells' in our models. The final goal of this study is to establish cell line-based, *in vitro* model systems specifically designed to investigate the relation between PI-TPα expression, on the one hand, and neuronal survival and neurodegeneration on the other.

Materials and methods

Rat-derived primary motor neuron and astrocyte cultures

Isolation of motor neurons from rodent spinal cord has been originally described by Schnaar and Schaffner in 1981 [13] and was based on the buoyancy of the motor neuron in a dense solution, which is much higher, because of its larger volume, compared to other populations of neurons. The dense solution used was metrizamide. Using different metrizamide percentages and embryos of different ages the procedure has been optimised by Camu and Henderson [14]. In the isolation procedure, during one step, glial cells are concentrated. If this particular fraction is isolated and cultured in the presence of serum, spinal cord-derived astrocyte cultures can readily be obtained.

Isolation of embryonic, rat spinal cord motor neurons and astrocytes

The method used to isolate embryonic, rat-derived rat spinal cord motor neurons and astrocytes has been described in detail in chapter 2. A summary of the isolation procedure is illustrated in Figure 3.1

NSC-34 cells, a mouse derived motor neuron-like cell line

To obtain a proliferating, uniform cell line that was still expressing a variety of motor neuron properties, Cashman and co-authors [10] fused mouse, spinal cord-derived, motor neurons with N18TG2 neuroblastoma (neuroblastoma x spinal cord or NSC cells). The obtained clones were tested for motor neuron properties. Clone 34 showed process extension, acetyl choline accumulation and release, choline acetyl transferase expression, action potential generation, induction of twitching of co-cultured muscle cells, neurofilament protein expression and expression of a receptor for the neuromuscular junction-specific basal lamina adhesion molecule (S-laminin).

Culture conditions for NSC-34 cells

Cells were grown in DMEM containing high glucose, pyruvate and glutamax (Gibco, no. 31966-021) supplied with 10% FBS (heat-inactivated, EU approved, Gibco no. 10108-165) and penicillin and streptomycin in a humidified atmosphere at 37°C, and 5% CO_2. To improve cell attachment, cells were grown in flasks and wells with a modified polystyrene surface (CELL BIND, Costar-Corning). Cells

were split approximately twice a week until passage 25-30. Cells were grown until 70-80% confluent. Occasionally, cells were cultured on glass cover slips in culture dishes. Although culturing NSC-34 cells in special CELL BIND culture plastics with a modified polystyrene surface greatly improved attachment, washing steps during experiments were avoided when possible to prevent cell loss.

C6 astroglioma cells, a rat-derived astrocyte cell line

C6 astroglioma cells were first described by Benda and collegues [11] and were derived from rat glial tumours induced by injection of N-Nitrosomethylurea in rat

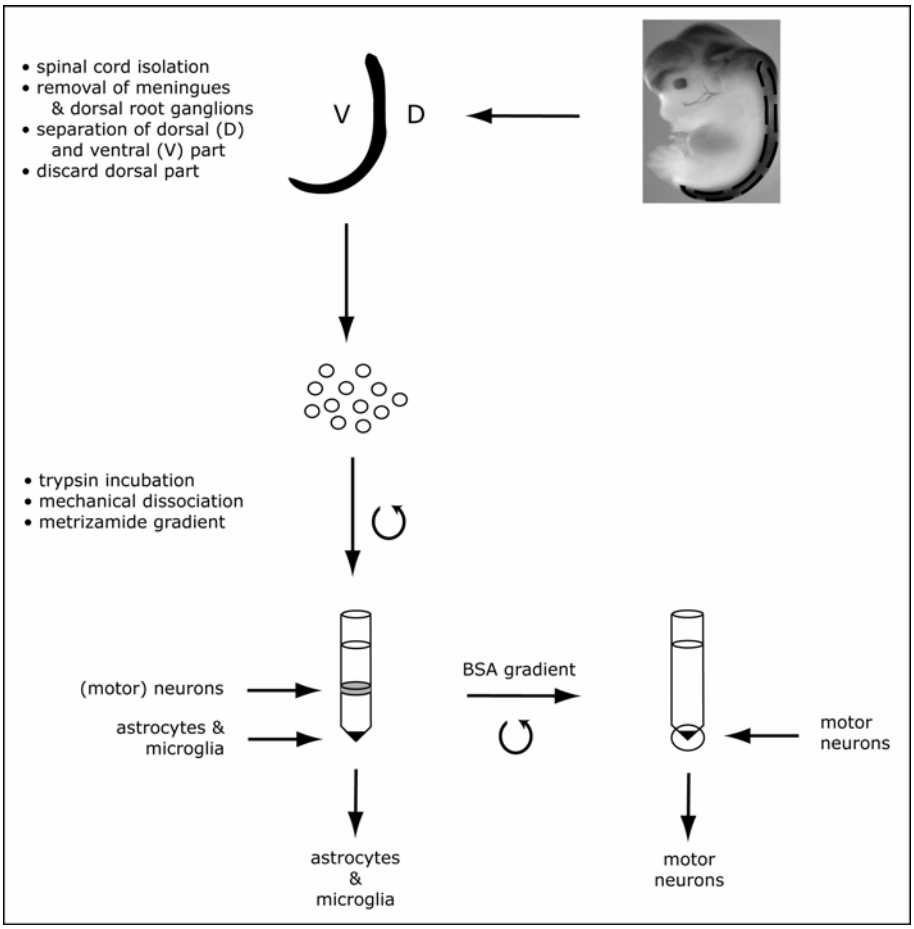

Figure 3.1: Scheme representing the isolation procedure of rat-derived embryonic motor neurons and glial cells.

brain. Tumours were grown in culture flasks and differentiated into astrocyte-like cells. Clone C-6 showed S-100 protein expression, which is considered to be an astrocyte-specific marker. S-100 expression increases as the cell grow from low density to a confluent cell layer, indicating that an astrocyte-like phenotype is reached upon confluency.

Culture conditions for C6 astroglioma cells

Cultures were grown in Ham's F10 medium containing glutamax (Gibco, no. 41550-021), supplemented with 10% FBS and penicillin and streptomycin in a humidified atmosphere at 37°C, and 5% CO_2. Cells were split twice a week upon confluency. Cells were used until passage 30-35.

NIH3T3, a mouse fibroblast cell line

The NIH3T3 mouse fibroblast cell line was established in 1963 by trypsinising and subsequently plating cells originating from whole Swiss mouse embryos (sacrificed at embryonic day 17-19) [15]. After a rapid decline in growth rate in different cultures, the growth rate gradually increased and cultures developed within three months. Spontaneously immortalised cells with stable growth rate were established after 20-30 generations in culture, and named '3T3' cells. The abbreviation 3T3 refers to the culturing protocol according to which the cells were continuously transferred (the 'T') every 3 days (the first '3'), and inoculated at the rigid density of 3 x 10^5 cells per 20 cm^2 dish (the second '3'). The 3T3 cell line showed a doubling time of approximately 24 hours. Upon confluency cell proliferation was halted, indicating a contact inhibition mechanism.

SPIβ cells, NIH3T3 cells overexpressing PI-TPβ

The cDNA encoding for mouse PI-TPβ was cloned into an expression vector (pBK-CMV) containing G418 resistance and a CMV promoter and subsequently transfected into NIH3T3 cells. After a selection procedure in which the death of non-transfected cells was triggered by the addition of the antibiotic G418, resistant clones with cells overexpressing PI-TPβ were obtained. RT-PCR and immunoblotting were used to detect respectively PI-TPβ mRNA and protein expression in NIH3T3 cells and SPIβ cells. Known PI-TPβ concentrations were used as a standard. The level of PI-TPβ protein expression was shown to be

upregulated 10-15 times in SPIβ cells [16]. Due to this high expression level, growth rate decreases compared to wild type fibroblasts (doubling times were 35 and 21 hours respectively). In addition, cell density was decreased upon confluency.

SPIα cells, NIH3T3 cells overexpressing PI-TPα

The cDNA encoding for mouse PI-TPα was cloned into an expression vector (pSG5) and was regulated by an SV40 promoter. This vector, together with a pSV2-neo vector providing G418 resistance, was transfected into NIH3T3 cells. After a selection procedure in which the non-transfected cells were eliminated by the addition of G418, resistant clones with cells overexpressing PI-TPα were obtained. Immunoblotting was used to detect the PI-TPα protein level in NIH3T3 cells and several clones overexpressing PI-TPα. Using known PI-TPα concentrations as a standard showed that SPIα cells show a two- to three-fold overexpression of protein PI-TPα [5].

In many aspects the phenotype and behaviour of SPIα cells mirrors the phenotype of SPIβ cells. As a result of PI-TPα overexpression, the doubling time of SPIα cells is decreased to 13 hours compared to 21 hours in wild type fibroblasts. In addition, cell density upon confluency is increased [5].

Culture conditions for NIH3T3 cells and SPIα cells

Cells were cultured in DMEM containing high glucose and glutamax (Gibco no. 61965-059), supplemented with newborn calf serum (NCS; 10%) and penicillin and streptomycin in a humidified atmosphere at 37°C, and 5% CO_2. Cells were split twice a week. SPIα cells were grown in G418 (400 μg/ml) to prevent disposal of the transfected vector.

Western blot analysis of PI-TPα expression

For the determination of PI-TPα expression, 80-100% confluent cell layers of different cell types were washed with PBS and subsequently lysed in ice-cold lysisbuffer containing 10 mM Tris (pH non-adjusted) and 0.1% Nonidet P-40 in de-mineralised water. After centrifugation for 10 min at 20,000 g (4°C), the protein concentration in the samples was determined using a Bradford assay [17]. A

Coomassie Brilliant Blue stained gel was analysed to verify the loading of equal amounts of protein. 20-30 μg aliquots of total protein were added to 5x Laemmli sample buffer [18], resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Different amounts of purified PI-TPα were loaded to use as a calibration curve. After blocking in non-fat milk powder for 1 h, membranes were incubated with an affinity-purified polyclonal rabbit antibody raised against synthetic peptides representing specific epitopes of PI-TPα [12] (1:500; 1 h). After washing, a goat-anti-rabbit antibody conjugated to horseradish peroxidase (1:5000) was used as a secondary antibody (1 h). Membranes were washed and developed with enhanced chemilumincence detection reagents according to the manufacturer's instructions. Quantification of bands on film was performed by scanning with a Bio-Rad GS 700 imaging densitometer equipped with integrating software.

Results

PI-TPα expression

The PI-TPα levels in the various cell lines were analysed by Western blot analysis and quantified using a calibration curve of purified PITPα. As shown in Figure 3.2, our data confirm that, similar to previous reports, the PI-TPα level in SPIα cells is approximately three times higher as compared to NIH3T3 cells [5]. Furthermore, as far as the different CNS-derived cell types are concerned, marked differences in the level of PI-TPα expression is observed between neuronal and non-neuronal cells, respectively.

As depicted in panel A of Figure 3.2, it was found that primary rat astrocytes express a relatively high level of PI-TPα which is in agreement with the immunocytochemical data previously presented in chapter 2. Compared with the expression in SPIα cells, the level of PI-TPα in primary rat astrocytes appeared to be approximately in the same range or might be even slightly higher. Panel B of Figure 3.2 shows the PI-TPα levels in the C6 astroglioma and NSC-34 cell lines. Like primary astrocytes, C6 astroglioma cells contain a relatively high level of PI-TPα. When compared to SPIα cells and primary astrocytes, the PI-TPα

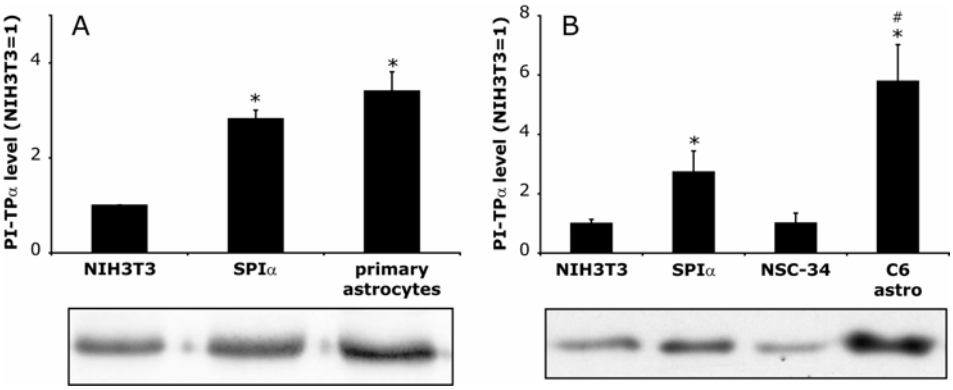

Figure 3.2: Western blots and corresponding graphical illustrations of quantified PI-TPα levels in different cell cultures. 30 μg (panel A) 20 μg (panel B) aliquots of total cell lysates were analysed. A Coomassie Brilliant Blue stained gel was analysed to verify the loading of equal amounts of protein. Panel **A** shows the PI-TPα levels in NIH3T3 mouse fibroblasts, NIH3T3 cells overexpressing PI-TPα (SPIα cells) and primary, spinal cord-derived astrocytes. Panel **B** shows, besides NIH3T3 and SPIα cells, the motor neuron cell line NSC-34 and the astrocyte cell line C6 astroglioma (C6 astro). Quantification of the obtained protein bands on film was performed by scanning with a Bio-Rad GS 700 imaging densitometer equipped with integrating software. Values were normalised to NIH3T3 cells. Western Blots are representative results of five independent experiments (primary astrocytes: three independent experiments). Values in the graph show averages ± SEM of 3 (panel A) or 5 (Panel B) independent experiments. $^{*}p<0.05$ vs. NIH3T3; $^{\#}p<0.01$ vs. SPIα.

expression levels in C6 astroglioma cells is found to be approximately two-fold higher.

In contrast to primary astrocytes and C6 astroglioma cells, the relative level of PI-TPα in primary motor neurons could not be quantified by Western blot analysis. Due to the fact that the cell density in these primary cultures is extremely low, in our hand it turned out to be rather difficult to obtain sufficient amounts of protein for SDS-PAGE. However, in the previous chapter we already presented evidence, albeit at the immunocyto-chemical level, for the expression of detectable levels of PI-TPα in primary motor neurons [9] (chapter 2). Here, we found that, similar to primary motor neurons, PI-TPα in the motor neuron cell line NSC-34 is expressed, however rather modest, i.e. being comparable to the level of expression in wild type NIH3T3 cells.

Discussion

In line with the observation that downregulation of PI-TPα results in the loss of motor neurons *in vivo*, we discovered that conditioned medium derived from a genetically-engineered fibroblast cell line overexpressing PI-TPα (i.e. SPIα cells) enhances the survival of motor neurons *in vitro*.

To further our studies into the relationship between PI-TPα-dependent survival factors and neuronal survival and neurodegeneration, in the current study we developed *in vitro* models based on CNS-derived cell lines. Ideally, such models will turn out to be very instrumental for our studies in that they allow large-scale biochemical analyses and experimental manipulation of PI-TPα expression, e.g. by using RNAi interference techniques.

To develop a cell line-based model we used the original, well-characterised NIH3T3/SPIα/SPIβ model as a starting point. Using this model, we previously showed in our laboratory that a relatively high PI-TPα level is required to become resistant towards induced apoptosis (i.e. enhance survival in an autocrine fashion) and to be capable to secrete survival factors (i.e. enhance survival in a paracrine fashion) [7]. To identify and select potential 'survival factor-producing cells' and 'target cells' with relevance for studies focussed on the role of PI-TPα in the CNS, the level of PI-TPα in different CNS-derived cell cultures was determined by Western blot analysis.

As far as the alternative 'target cell' is concerned, we found that PI-TPα expression in the motor neuron-like NSC-34 cell line was relatively low, which is in agreement with data previously shown for the neuronal PC12 cell line [6]. The above observation, together with its well-documented relevance for studies into the pathophysiology of motor neurons, led us conclude that the NSC-34 cell line may well serve as a 'target cell' in our CNS-dedicated model system. Previously, both SPIβ and primary motor neuron cell cultures have been proven to be suitable read-out systems to demonstrate the survival-enhancing effects of PITPα-dependent factors derived from PI-TPα expressing cells. Whether or not NSC-34 cells are equally sensitive to the survival-promoting effect of PI-TPα-dependent factors, and thus can be used as an alternative 'target cell' in our models remains an open question that will be addressed in the experiments described in chapter 5.

As mentioned already in the introduction it is tempting to hypothesize that non-neuronal cells might somehow be implicated in the production of PITPα-dependent survival factors in the CNS. In line with our expectations, and in line with the immunocytochemical observations presented in chapter 2, it was found that both primary astroglial cells and the C6 astroglioma cell line showed a relatively high level of PI-TPα expression. Therefore astrocytes are considered to be good alternatives to fulfil the role of 'survival factor-producing cells' in our model. Although at this stage the potential protective effect of other cell types in the CNS can not be excluded, at least the fact that PI-TPα levels in both primary astrocytes and C6 astroglioma are equal or even considerably higher when compared with the level of PI-TPα in SPIα cells, appears in line with the above postulated hypothesis concerning the role of astroglial expression of PI-TPα and neuronal survival. It should be noted, however, that such critical questions as to whether astroglial cells are indeed capable of the production and release of survival-enhancing factors, as previously demonstrated for SPIα cells, and whether or not this phenomenon is correlated to cellular PI-TPα protein expression, cannot be answered from the data presented in the present study. These issues, that are central to the scientific aim of this thesis, will be addressed in chapter 4 and 5, respectively.

The stepwise metamorphosis of the original NIH3T3/SPIα/SPIβ model [7] is summarised in Figure 3.3. First, as illustrated in panel A and described in chapter 2, SPIβ 'target cells' were replaced by primary motor neurons thus creating the model in which the neuroprotective efficacy of SPIα could be established. Secondly, to specifically investigate the role of astrocytes as potential 'survival factor-producing cells', in the model depicted in panel B the SPIα cells were substituted for primary astrocyte or C6 astroglioma cultures, respectively. At the same time the well-characterised and validated SPIβ 'target cell' was left unchanged for sake of comparison. This latter model will be used in chapter 4 and 5. Finally, as shown in panel C and as described in chapter 5, next to SPIβ cells, NSC-34 cell cultures are included as 'target cell', thus creating a complete cell line-based model mimicking neuro-glia interactions in the CNS. As indicated in panel B and C, to manipulate PI-TPα protein expression in C6 astroglioma, RNAi approaches can be considered.

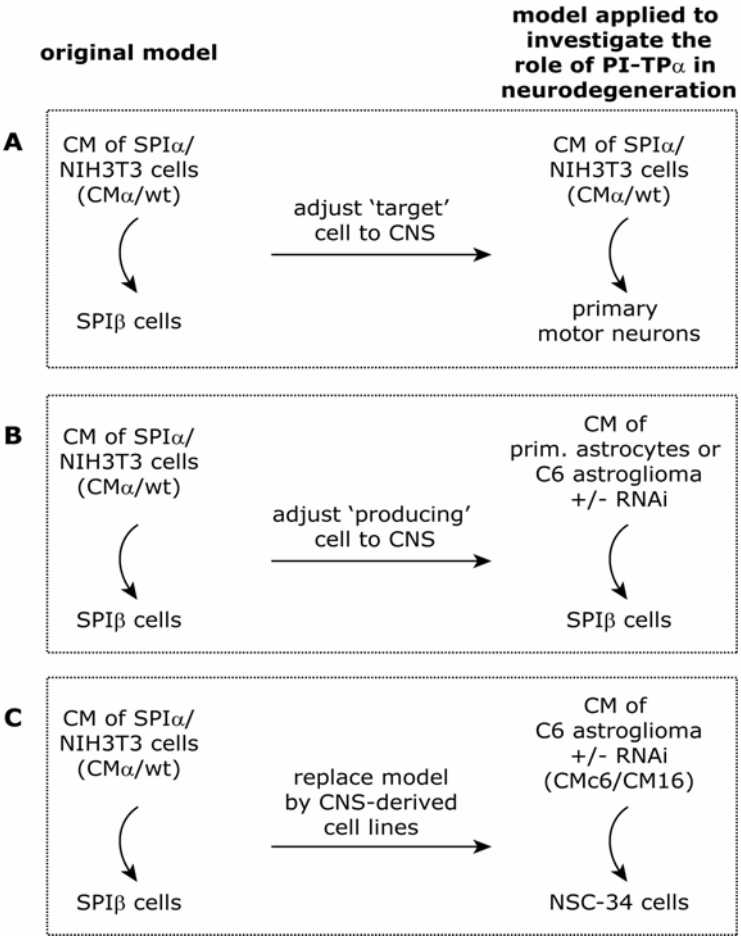

Figure 3.3: Evolution of the original cellular model, which was generated to investigate PI-TPα function in general, towards a CNS-derived *in vitro* model specifically adjusted to investigate the role of PI-TPα in neurodegeneration. The left side of each panel shows the original model in which the PI-TPα-derived anti-apoptotic factor was investigated in general: NIH3T3 cells overexpressing PI-TPα (SPIα cells) as factor-producing cells and NIH3T3 cells overexpressing PI-TPβ (SPIβ cells) as sensitive target cells. In panel **A**, the SPIβ cells were substituted for primary motor neuron cultures to investigate the neuroprotective activity of the PI-TPα-dependent factor(s) (chapter 2) In Panel **B**, the SPIα cells were substituted for primary astrocytes and C6 astroglioma cells. As demonstrated in chapter 5, in the latter cell line, it is relatively straightforward to apply the RNA interference technique to test the PI-TPα-dependence of the observed neuroprotective effects. In panel **C**, the original model as a whole is replaced by CNS-derived cell lines (i.e. C6 astrocytoma cells as 'factor-producing cells' and the NSC-34 motor neuron cell line as 'target cells'), obtaining a CNS-derived *in vitro* model to investigate the role of PI-TPα in neurodegeneration.

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Chapter 4:

Characterisation of the effect of conditioned medium from C6 astroglioma cells on the survival of serum-deprived SPIβ cells

Hanneke Bunte, Martijn Schenning, Karel W.A. Wirtz, Dop P.R. Bär, Gerry T. Snoek, and Freek L. van Muiswinkel

Abstract

Selective neuronal loss is a prominent feature in both acute and chronic neurological disorders. Recently, reduced expression of the highly conserved phosphatidylinositol transfer protein α (PI-TPα) in experimental mouse models has been related among others to severe neurodegeneration of the motor system. On the other hand, increased expression of PI-TPα in cultured mouse fibroblasts resulted in increased resistance towards induced apoptosis and the secretion of survival-enhancing factors, capable of protecting primary motor neurons *in vitro*.

Since PI-TPα expression was found to be high in astrocytes, it was investigated whether the conditioned medium of these cells exhibit survival-enhancing activity. We show that conditioned medium derived from both primary astrocytes and C6 astroglioma cells (CMc6) enhance the survival of serum-deprived apoptosis-sensitive SPIβ cells. Since a lipid extract derived from these conditioned media is also protective, we suggest that a lipid factor is involved in the survival-enhancing effect. This effect is markedly reduced when CMc6 is co-incubated with an antagonist of the cannabinoid-1 receptor SR141716A (rimonabant), thus indicating that this receptor might be involved. Liquid chromatography coupled to mass spectrometry analysis of total lipid extracts of CMc6 showed a decrease in eicosanoid precursors, indicating a high eicosanoid metabolism. Moreover, comparison of total lipid extracts of CMc6 with CM of fibroblasts overexpressing PI-TPα, by HPLC coupled to mass spectrometry, revealed three PI-TPα- and cyclooxygenase-2-dependent peaks present in both media that co-eluted in the range of the endocannabinoids.

In summary, these data imply that astrocytes may produce and secrete endocannabinoid-like lipid factors that are capable of protecting apoptosis-sensitive cells against serum-deprivation-induced cell death, possibly via the cannabinoid-1 receptor.

Introduction

The highly conserved phosphatidylinositol transfer protein α (PI-TPα) has been identified in many different tissues derived from mammals, plants, fungi and yeast [1]. In addition, several high molecular weight, membrane-bound, proteins with PI-TPα-related domains have been reported (e.g. RdgB and Nir proteins) [2, 3]. By analysing mouse tissues, it was shown that PI-TPα expression is highest in the central nervous system (CNS) [4]. *In vitro*, PI-TPα is capable of transferring phosphatidylinositol (PI) and phosphatidylcholine (PC) between membranes [5].
In vivo studies in fly, mouse and zebrafish revealed that a decrease in the expression of PI-TPα or PI-TPα-related proteins causes severe and progressive neurodegeneration. Thus, null mutations in RdgBα (a protein with a domain showing 40% identity with PI-TPα) in *drosophila* result in light-enhanced degeneration of the photoreceptor cells in the retina [2]. In 1997 it was found that the vibrator mouse, suffering from progressive action tremor caused by degeneration of neurons in the cerebellum, brainstem, and spinal cord, showed an 80% reduction of PI-TPα protein expression [6]. Moreover, PI-TPα knock-out mice die within 14 days after birth due to progressive spinocerebellar degeneration [7]. Finally, Xie and co-workers [8] found, analysing zebrafish embryos in which PI-TPα was downregulated, that PI-TPα is involved in neurite outgrowth in spinal cord motor neurons. These data indicate an important role of PI-TPα in the development and maintenance of neuronal cells.
Analysis of the NIH3T3 mouse fibroblast cell line overexpressing PI-TPα (sense PI-TPα cells or SPIα cells) revealed that overexpression of PI-TPα resulted in the enhanced production of arachidonic acid, as well as arachidonic acid metabolites, possibly derived from PI. In addition, the expression of cyclooxygenase-2 (COX-2) was upregulated in these cells [9, 10]. SPIα cells are extremely resistant against UV-induced apoptosis when compared to wild type NIH3T3 cells. Interestingly in wild type NIH3T3 cell cultures, conditioned medium from SPIα cells (CMα) was shown to confer protection against apoptosis induced by UV radiation, serum deprivation, or TNFα treatment [4]. This protection is most likely attributable to the secretion and action of a COX-2-dependent arachidonic acid metabolite(s), since the protective activity of CMα was reduced by a COX-2 inhibitor [10]. Furthermore, it was shown that the bio-active factors involved act via a G-protein

coupled receptor (GPCR), presumably the cannabinoid-1-receptor (CB1R) [10]. Interestingly, we have similarly shown that CMα was also capable of protecting primary, spinal cord-derived, rat motor neurons against serum deprivation-induced cell death. Again, the protection was partially dependent on the activation of a GPCR [11].

Since (i) expression of PI-TPα is highest in the CNS, (ii) reduction of PI-TPα results in neurodegeneration and reduced neurite outgrowth, and (iii) cells overexpressing PI-TPα (SPIα cells) produce and secrete a bio-active factor that confers neuroprotection *in vitro*, we hypothesise that in the CNS contains cells, the PI-TPα level of which may control neuron maintenance.

In chapter 3, it was shown that astroglial cell types (primary cultures as well as cell lines) show relatively high expression of PI-TPα. High expression of PI-TPα appears to be the first condition for producing the bio-active factor.

In addition to their high expression level of PI-TPα, astrocytes are (i) the most abundant glial cell type in the CNS, (ii) known to express COX-2 and PLA_2 [12, 13], (iii) associated with protective activity towards neurons [14-17] and known to increase motor neuron survival in motor neuron-astrocyte co-cultures *in vitro* [18]. Finally (iv) astogliosis (i.e. astrocyte proliferation) has been implicated in the pathogenesis of many neurodegenerative diseases [19]. Therefore, we hypothesise that astrocytes might be a likely source for the endogenous production and secretion of PI-TPα-dependent neuroprotective factors within the CNS.

Since both primary astrocytes and the well-characterised astrocyte cell line C6 astroglioma contain a high expression level of PI-TPα (chapter 3), we have analysed the effect of conditioned medium derived from both cell cultures on the survival of the apoptosis-sensitive SPIβ cells. In addition, we have characterised the mode of action of the conditioned medium of C6 astroglioma cells (CMc6) in more detail. Using receptor antagonists of the CB1- and CB2R and the broad-spectrum GPCR inhibitor suramine, it was investigated whether activation of GPCR and/or cannabinoid receptors are involved in the protective effect of CMc6 on SPIβ cells. In addition, the levels of arachidonic acid and other eicosanoid precursor molecules in CMc6 were determined and compared to the levels in CMα. Finally, to gain further insight into the nature, and in an attempt to identify the chemical entity of the factors responsible for the survival-enhancing effect, the PI-TPα- and COX-2-dependent compounds present in total lipid extracts from

CMα and CMc6 were compared using high performance lipid chromatography coupled to mass spectrometry (HPLC/MS).

Materials and Methods

Chemicals

Culture media and sera (Gibco), NS-398 (Cayman), PARP antibody (Santa Cruz), Cleaved caspase-3 antibody (Cell signaling technology), monoclonal β-actin antibody (Sigma), ethyl acetate (Merck), formic acid (Merck), ethanol absolute (Merck), suramin (Sigma), goat-anti-rabbit horseradish peroxidase antibody (Biorad), goat-anti-mouse horseradish peroxidase antibody (Biorad), Bradford assay (Biorad) and enhanced chemilumincence detection reagents (Amersham Biosciences) were obtained from the suppliers indicated.

Cell culture

NIH3T3 cells overexpressing PI-TPα (SPIα cells) and PI-TPβ (SPIβ cells) were made as described previously [9, 20]. NIH3T3 cells, SPIα and SPIβ cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose and glutamax, supplemented with newborn calf serum (NCS; 10%) and penicillin and streptomycin in a humidified atmosphere at 37°C, and 5% CO_2. Cells were split once (SPIβ) or twice (NIH3T3 and SPIα) a week. SPIα and SPIβ cells were grown in G418 (geneticin; 400 μg/ml) to prevent disposal of the transfected vector. During experiments G418 was excluded from the medium.
C6 astroglioma cell cultures [21] were grown in Ham's F10 medium containing glutamax, supplemented with 10% FBS and penicillin and streptomycin in a humidified atmosphere at 37°C, and 5% CO_2. Cells were split twice a week upon confluency. Cells were used until passage 30-35.

Preparation of conditioned media (CM) and lipid extracts

CM of NIH3T3 and SPIα cells was prepared as described previously [10]. Briefly, cell cultures of either wild type NIH3T3 mouse fibroblasts or SPIα cells were grown to 80-90% confluence in DMEM containing 10% newborn calf serum.

After washing twice with phosphate buffered saline (PBS; pH 7.4), the medium was replaced by serum free DMEM without phenol red containing glutamax and 0.1% BSA (DBB). After 24 h, the medium was collected and centrifuged (5 min at 500 g) to yield either CMα or CMwt (from SPIα or wild type NIH3T3 cells, respectively). To obtain CMα in which cyclooxygenase-2-dependent metabolites were reduced, the COX-2 inhibitor NS-398 (50 μM) was added to the DBB medium.
To obtain CM from C6 astroglioma cells, cells were plated in a 10-cm dish (55 cm^2) with a cell density of $3.4x10^6$ cells per dish. After 4 days, the confluent cell culture was washed twice with PBS. Medium was replaced with DBB and after 24 h, the resulting CM was collected and centrifuged (5 minutes at 500 g) to yield CMc6. Similar to SPIα cells, a confluent cell layer of C6 astroglioma cells was required for an optimal production/secretion of the survival-enhancing factor(s) (data not shown).
All conditioned media were stored at 4^oC until further use.
Lipid extracts, containing arachidonic acid metabolites, were obtained from DBB, CMc6, CMα, and CMwt as described previously [22]. Briefly, after addition of 30 μl of 12 M formic acid to one ml of CM, the mixture was extracted with two 3 ml volumes of ethyl acetate. The combined extracts were then evaporated under nitrogen gas before the residues were dissolved in small aliquots of ethanol, which were diluted in DBB to appropriate concentrations (an extract of x ml CM was redissolved in x ml DBB) and immediately used after preparation. During the experiments ethanol concentrations never exceeded 0.2%).

Induction of apoptosis in SPIβ cells by serum deprivation

SPIβ cells are very sensitive to apoptosis induced by e.g. UV radiation, TNFα incubation, or serum deprivation [4]. Protection of SPIβ cells against serum deprivation-induced apoptosis by the CM of SPIα cells (CMα) has already been described [4]. In short, SPIβ cells were grown until 95-95% confluent in normal growth medium without G418. Cells were washed with PBS twice to remove remaining serum. CM or a LE was added at the appropriate concentration (i.e. 1 cm^2 of SPIβ cells was incubated with CM or LE derived from 1 cm^2 of cells, diluted in DBB). DBB served as a control. To investigate the involvement of CB1

or CB2 receptors, 1 μM of SR141716A (rimonabant, acomplia™) or SR144528 (inhibitors of CB1 and CB2 receptors respectively [23]) was added to the medium before adding the mixture to the cells. After 8 h of incubation, cells were photographed using a light microscope and washed twice with ice-cold PBS. Medium and PBS were collected and centrifuged at 4°C (5 minutes at 500g) to collect floating cells. Pellets were washed with ice-cold PBS. Cells and cell pellets were stored at -20°C until further processing.

Analysis of PARP in treated SPIβ cells

Frozen SPIβ cells and cell pellets obtained after induction of apoptosis by serum deprivation were lysed on ice in ice-cold lysisbuffer (containing 10 mM Tris and 0.1% Nonidet P-40) and centrifuged for 10 min at 20,000 g (4°C). After determining the protein concentration using a Bradford assay [24], 40 and 60 μg aliquots of total protein were prepared in 5x Laemmli sample buffer [25] for the analysis of poly(ADP-ribose) polymerase-1 (PARP).

Proteins were resolved on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Subsequently blots were cut to separate (cleaved) PARP (85/116 kD) and β-actin (43 kD). After blocking in non-fat milk powder (1 h), membranes were incubated with anti-rabbit PARP antibodies (1:1000), or anti-mouse β-actin antibodies (1:2000) in diluted non-fat milk powder overnight. After washing, a goat-anti-rabbit or mouse antibody conjugated to horseradish peroxidase (1:5000) was used as a secondary antibody (1 h). Blots were washed and developed with enhanced chemiluminescence detection reagents according to the manufacturer's instructions. Quantification of the bands was performed by scanning the film with a Bio-Rad GS 700 imaging densitometer equipped with integrating software. Experiments were performed in duplicate.

Extraction of fatty acid metabolites for MS analyses

To extract all fatty acids secreted during the production of CMwt, CMα and CMc6, the conditioned media were subjected to methanol/chloroform (1:2, v/v) extraction [26, 27]. The organic phase was dried under nitrogen gas and reconstituted in chloroform/methanol (1:4, v/v) for further analysis. Extraction of DBB served as a control.

Fatty acid metabolite analyses by liquid chromatography/mass spectrometry (LC/MS)

Extracted fatty acid metabolites were analysed using an 1100-LC system coupled to a 1946A-MS detector (Agilent Technologies, Palo Alto, CA) equipped with ESI interface. XDB Eclipse C18 column (50 x 4.6 mm ID, 1.8 μm, Zorbax), eluted with a gradient of methanol in water (from 0% to 100% methanol in 2.5 min) at a flow rate of 1.5 ml/min. Methanol contained 0.25% acetic acid and 5 mM of ammonium acetate. Column temperature was kept at 40°C. MS detection was performed in the positive ionisation mode; capillary voltage was set at 3 kV. Nitrogen was used as drying gas at a flow rate of 13 L/min and a temperature of 350°C. Nebulizer pressure was set at 60 psi.

Fatty acids were quantified using an isotope-dilution method [28], which monitors sodium adducts of the molecular ions $[M+Na]^+$ in the selected ion-monitoring (SIM) mode.

Fatty acid metabolite analyses by high performance lipid chromatography coupled to mass spectrometry (LC/MS)

The system used for HPLC/MS was identical to that used for LC/MS (see above). CM was subjected to methanol/chloroform (1:2, v/v) extraction, and separated using a XDB Eclipse C18 column. Subsequently elution occurred using a gradient of methanol in water (from 60% to 100% methanol in 15 min; 5 min at 100% methanol; from 100% to 60% methanol in 1 min) at a flow rate of 1 ml/min. Methanol contained 0.25% acetic acid and 5 mM of ammonium acetate. Column temperature was kept at 30°C. MS detection was performed in the positive ionisation mode; full scan from 250 m/z to 500 m/z; capillary voltage was set at 3 kV. Nirogen was used as drying gas at a flow rate of 13 L/min and a temperature of 350°C. Nebulizer pressure was set at 60 psi.

Results

The effect of the conditioned medium of C6 astroglioma cells (CMc6) on induction of apoptosis in SPIβ cells by serum deprivation

To investigate whether C6 astroglioma cells produce and secrete (a) bio-active factor(s) capable of enhancing cell survival, conditioned medium of these cells

(CMc6) was tested on SPIβ cells grown in the absence of serum. The cells were incubated with DBB or CMc6 for 8 hours to induce serum deprivation-induced cell death. Cells incubated with CMwt or CMα served as a control. Figure 4.1A-D show images of SPIβ cells after 8 hours of serum deprivation in the presence of DBB (panel A), CMwt (B), CMα (C) or CMc6 (D). The appearance of 'blebbing' structures on the plasma membrane of the cells, as an indication of apoptosis [29], is frequently observed when cells were incubated with DBB or, to a lesser extent, with CMwt. This morphological indication of apoptosis is almost absent when cells were incubated with CMc6 or CMα. Morphological scoring of blebbing SPIβ cells after incubation with the different media revealed that approximately 50% of the cells were found to be blebbing cells in DBB, 20% in CMwt, and approximately 5% in CMα and CMc6.

This observation is biochemically confirmed by detecting poly(ADP-ribose) polymerase-1 (PARP) cleavage in cell lysates of SPIβ cells 8 hours after incubation with the different conditioned media or DBB. PARP (113 kD) is cleaved into two fragments (89 and 24 kD) by caspases, enzymes that become activated upon cleavage after the induction of apoptosis [30]. Figure 4.1E shows that, similar to

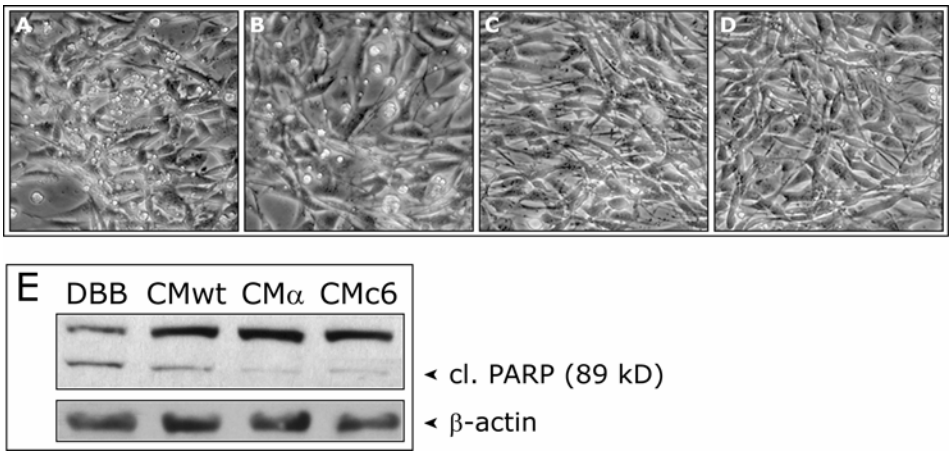


Figure 4.1: Conditioned medium of C6 astroglioma cells (CMc6) enhances survival of SPIβ cells in the absence of serum. Panel **A-D** show images of SPIβ cells 8 hours after the induction of serum deprivation. Cells are incubated with serum free medium (DBB, panel A), CMwt (B), CMα (C) or CMc6 (D). Panel **E** shows a Western blot of SPIβ cell lysates after 8 hours of serum deprivation in different CM. The blot was immunostained with antibodies against PARP to determine the level of cleaved (cl.) PARP and hence the amount of apoptotic cells, and with β-actin to confirm equal protein loading. Images and Western blot are representative results of four independent experiments.

the effect of CMα, PARP cleavage is significantly decreased upon incubation with CMc6 compared to incubation with DBB or CMwt. Compared to DBB, the cleaved PARP/actin ratios of SPIβ cell lysates incubated with CMwt, CMα and CMc6 were decreased by approximately 70%, 95% and 90%, respectively.
Comparable to CMc6, the conditioned medium of primary, spinal cord-derived astrocytes is also capable of enhancing the survival of SPIβ cells in the absence of serum, based on morphological scoring (data not shown).

The effect of a lipid extract of CMc6 on induction of apoptosis in SPIβ cells by serum deprivation

Previously, the cell survival-enhancing activity of the conditioned medium of astrocytes has been reported to be dependent on polypeptide growth factors [31] and protein- or peptide-related (precursors of) antioxidants [32]. To investigate whether the observed survival-enhancing activity of CMc6 is also present in a lipid extract as previously demonstrated for CMα [10], a lipid extract of CMc6 was

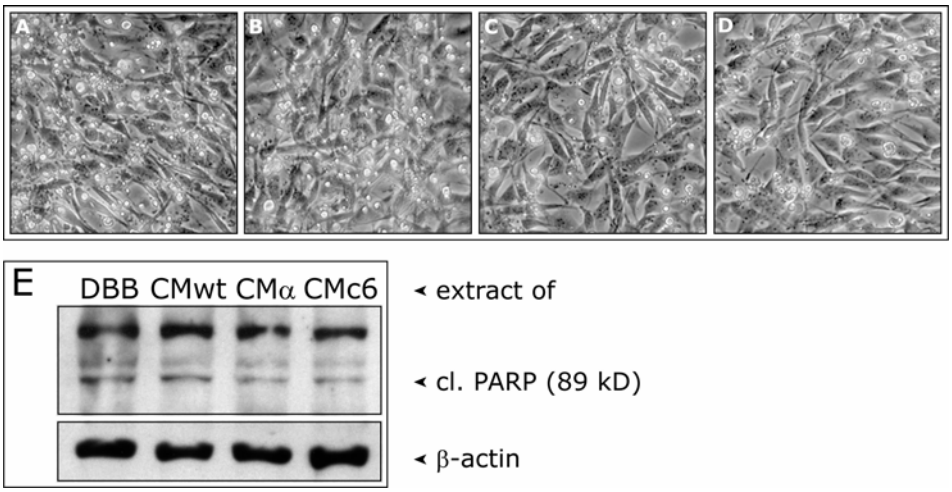


Figure 4.2: A lipid extract, containing arachidonic acid metabolites, of CMc6 enhances survival when incubated with SPIβ cells in the absence of serum. Panel **A-D** show images of SPIβ cells 8 hours after the induction of serum deprivation. Cells are incubated with extracts of DBB (panel A), CMwt (B), CMα (C) or CMc6 (D). Panel **E** shows a Western blot of SPIβ cell lysates after 8 hours of serum deprivation in extracts of different CM. The blot was immunostained with antibodies against PARP to determine the level of cleaved (cl.) PARP and hence the amount of apoptotic cells, and with β-actin to confirm equal protein loading. Images and Western blot are representative results of three independent experiments.

prepared and incubated with SPIβ grown cells in the absence of serum. Figure 4.2A-D show SPIβ cells 8 hours after incubation with a lipid extract from DBB (panel A), CMwt (B), CMα (C) or CMc6 (D). Fewer blebbing cells can be observed when SPIβ cells were incubated with the lipid extract from CMc6 (and CMα), indicating that lipid factors, among which arachidonic acid metabolites, contribute to the protective activity of CMc6. The morphological observation of the decrease in the amount of blebbing SPIβ cells is corroborated by a decrease in PARP cleavage (Figure 4.2E). The cleaved PARP/β-actin ratio decreases approximately 10% and 30% for the lipid extract of CMα and CMc6, respectively, compared to the lipid extract of DBB. No decrease in PARP cleavage was observed when SPIβ cells were incubated with a lipid extract of CMwt.

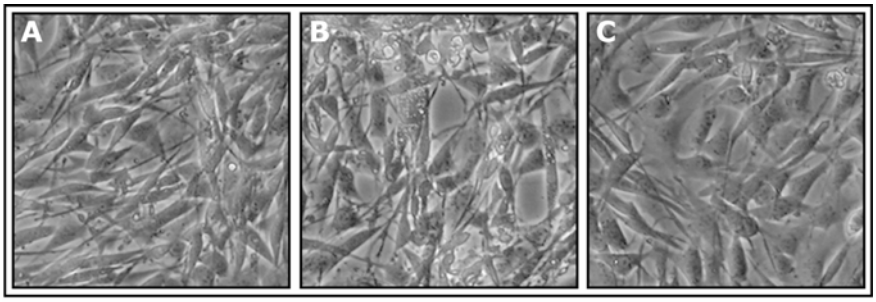
The effect of cannabinoid receptor antagonists on CMc6-induced protection of serum-deprived SPIβ cells

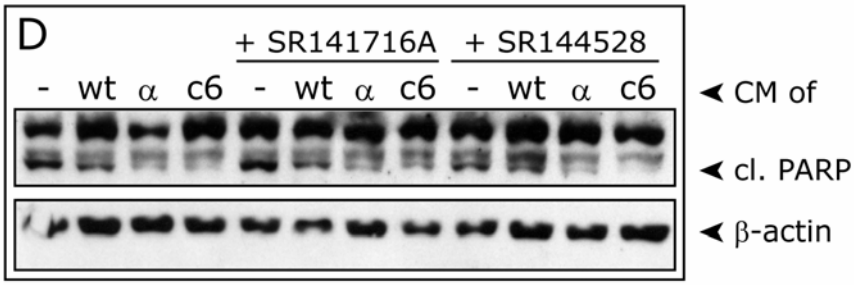
Previous studies showed that the bio-active factor(s) in CMα act via a GPCR, most likely the CB1R [10, 11]. Therefore it was tested whether co-incubation of CMc6 with SR141716A (rimonabant, acomplia™), a CB1R antagonist, influences the survival-enhancing effect of CMc6 on SPIβ cells grown in the absence of serum. In addition, the effect of co-incubation of SR144528, a CB2R antagonist, was investigated. Figure 4.3A and B show that, when CMc6 is administered in the presence of SR141716A (panel B), blebbing of SPIβ cells after 8 hours of serum deprivation is increased as compared to blebbing of SPIβ cells in the presence of CMc6 alone (panel A). Co-incubation with SR144528 (panel C) showed no changes in protection of SPIβ cells by CMc6, thus indicating that the CB1R, but not the CB2R, might be involved in the survival-enhancing effect of CMc6. Western Blotting and subsequent immunostaining with anti-PARP antibodies to determine the level of cleaved PARP (Figure 4.3D and the accompanying graph) confirm that the CB1R might be involved in the protection of CMc6 against apoptosis. Hence, when SPIβ cells are co-incubated with SR141716A and CMc6, the cleaved PARP/actin ratio increases 5.4-fold as compared with controls that were incubated with CMc6 alone. The ratio found in CMc6/SR141716A treated cells resembles the ratio found in cells incubated with CMwt. Similarly, when SPIβ

cells were co-incubated with CMα and SR141716A, the cleaved PARP/actin ratio increased 4.2-fold as compared to cell treated with CMα alone.
Relatively small, not statistically significant effect on the cleaved PARP/actin ratios were observed when SR141716A was added to DBB or CMwt (0.88- and 1.3-fold increase compared with the incubation without an antagonist, respectively). Remarkably, Co-incubation of SR144528 with DBB resulted in a marked protective effect towards SPIβ cells (the cleaved PARP/actin ratio was decreased by 60%) as previously shown by [10]. The other ratios, however, were virtually unchanged compared to incubation without an antagonist (a 0.98, 0.87, or 1.0-fold increase for co-incubation with CMwt, CMα, or CMc6, respectively), indicating that the CB2R is not likely to be involved in the survival-enhancing effect of either CMc6 or CMα. In agreement with these results it was shown that co-incubation of CMc6 or CMα with suramin, a broad-spectrum, non-specific antagonist of GPCRs [33], provided a similar outcome (data not shown), implying that the action of CMc6 is indeed (partially) established via a GPCR, presumably the CB1 receptor.

Detection of eicosanoid precursors in CMwt, CMα and CMc6

Arachidonic acid metabolites belong to the class of eicosanoids. Precursors for these compounds are, besides arachidonic acid, eicosapentaenoic acid and dihomo-γ-linolenic acid. As shown in Figure 4.4A, all three eicosanoid precursors are derived from linoleic acid via multi-reaction steps (dashed lines in panel A). Furthermore, dihomo-γ-linolenic acid can be converted to arachidonic acid [34, 35].
Figure 4.4B-D shows the levels of arachidonic acid (panel B), linoleic acid (C) and eicosapentaenoic acid (D) in a total lipid extract (chloroform/methanol extraction) of CMα, CMwt, CMc6 and DBB. As expected, serum free medium (DBB) does not contain any of the eicosanoid precursors. Interestingly, the levels of the secreted precursors are similar in the CM of SPIα cells and C6 astroglioma cells. Furthermore, these levels are consistently lower compared to the levels in CMwt. This indicates an active metabolism of the precursors of eicosanoids in SPIα and C6 astroglioma cells, confirming previous observations in SPIα cells [10].





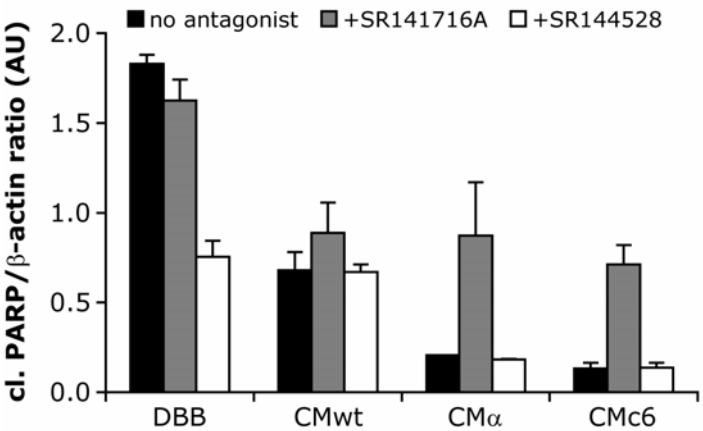


Figure 4.3: Co-incubation of CMc6 with an antagonist of the cannabinoid-1 receptor (CB1R), but not an antagonist of the CB2R, reduces the survival of SPIβ cells. Panel **A-C** show SPIβ cells after 8 hours of serum deprivation in CMc6 (A), CMc6+SR141716A (CB1R antagonist, panel B), or CMc6+SR144528 (CB2R antagonist, panel C). Panel **D** shows a Western blot of SPIβ cell lysates after 8 hours of serum deprivation in DBB (-), CMwt (wt), CMα (α) or CMc6 (c6) with or without SR141716A or SR144528. The blot was immunostained with antibodies against PARP to identify the amount of cleaved (cl.) PARP and thereby the levels of apoptotic cells, and with β-actin to confirm equal protein loading. Cl. PARP/ β-actin ratios, obtained by scanning of the Western blot in panel D with a Bio-Rad GS 700 imaging densitometer equipped with integrating software, are depicted in the graph. Images and Western blot are representative results of two independent experiments. The graph shows averages ± SEM of two independent experiments performed in duplicate.

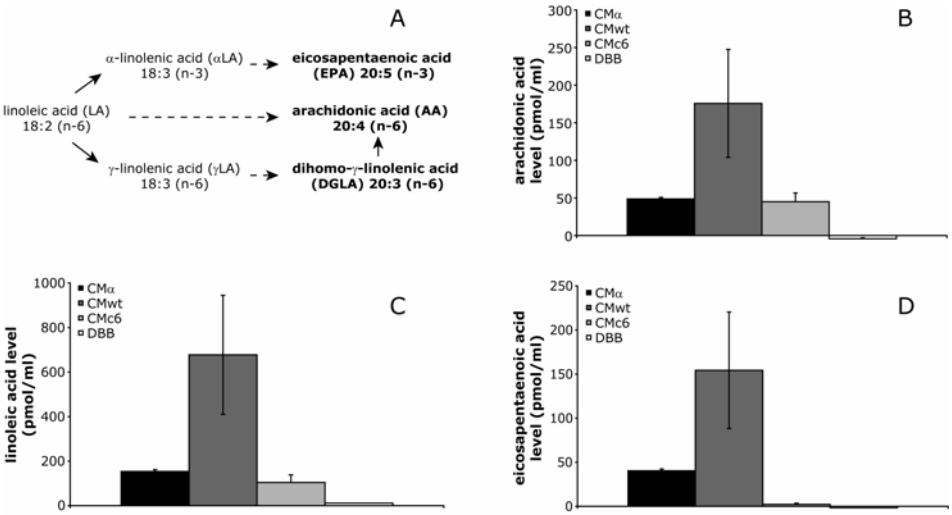


Figure 4.4: Levels of eicosanoid precursors in CMwt, CMα, CMc6, and DBB as measured by liquid chromatography coupled to mass spectrometry. Panel **A** shows a highly simplified synthesis pathway of the eicosanoid precursors arachidonic acid, eicosapentaenoic acid and dihomo-γ-linolenic acid. Arrows with dashed lines indicate multi-reaction steps. Panel **B-D** show the levels of arachidonic acid (B), linoleic acid (C) and eicosapentaenoic acid (D) in CMα, CMwt, CMc6 and DBB. Bars represent average values ± SD of three independent batches of CM.

Detection of changes in fatty acid metabolites in CMwt, CMα, and CMc6 by mass spectrometry

To gain further insight into the nature of the survival-enhancing factor(s) in CMc6, total lipid extracts (LE) of CMwt, CMα and CMc6 (LEwt, LEα, and LEc6, respectively) were analysed by high performance lipid chromatography coupled to mass spectrometry (HPLC/MS).

Since a lipid extract of CMwt does not show any survival-enhancing activity in SPIβ cells (Figure 4.2E), it may be assumed that the survival-enhancing factor in CMα and CMC6 is not present in LEwt. Based on this assumption, it should be possible to identify potential survival factors by comparing the total lipid extracts of the three media. In principle, compounds both present in both LEα and LEc6, but not in LEwt, would be candidate survival factors. As an example, Figure 4.5 shows the mass spectra of LEc6 and LEwt. Subtracting the total mass spectra of LEc6 and LEwt, respectively, yielded peaks (i.e. compounds) that are present in LEc6, but not in LEwt. By a similar subtraction of LEα and LEwt, those compounds that are only present in LEα can be identified. The remaining

common peaks, i.e. present in both (LEα - LEwt) and (LEc6 - LEwt), may be likely candidates for the survival-enhancing effect of CMα and CMc6.
On the other hand, previous studies showed that the anti-apoptotic activity of CMα is reduced when CMα was prepared in the presence of a COX-2 inhibitor ($CM\alpha^-$) [10]. Peaks that are not present in the mass spectrum of the total lipid extract of $CM\alpha^-$ ($LE\alpha^-$), but are present in the spectra of LEα and LEc6, are presumably COX-2-dependent products and therefore likely to be responsible for the survival-enhancing activity of CMα and CMc6. The final analysis resulted in three peaks that were present in both CMα and CMc6 but not in CMwt and $CM\alpha^-$. Table 4.1 shows the mass/charge (m/z) ratios of these peaks and their corresponding HPLC-related retention times. In addition the m/z ratio and retention time of several standard compounds are listed. The m/z ratios and retention times, especially of peak 2 and 3, closely resemble these of the endocannabinoids 2-arachidonyl glycerol and anandamide. Retention times and

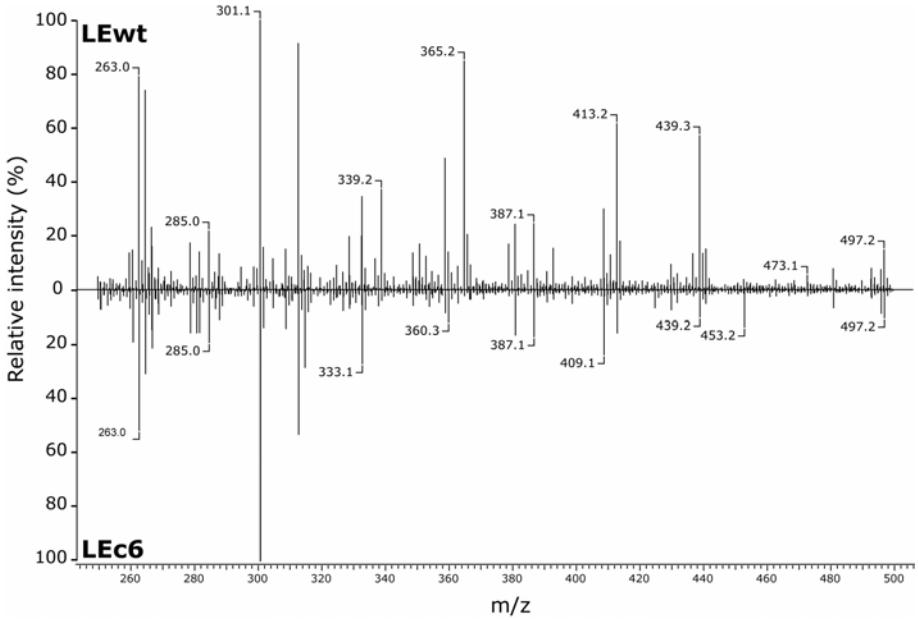


Figure 4.5: Total mass spectra between m/z (mass/charge) ratio 250 and 500 of total lipid extracts of CMwt (LEwt) and CMc6 (LEc6) analysed by high performance lipid chromatography coupled to mass spectrometry (HPLC/MS). CMwt and CMc6 were subjected to chloroform/methanol extraction as described in the materials and methods section and analysed by LC/MS. Intensities of m/z ratios of compounds present in LEwt are shown in the positive mode and compounds present in LEc6 in the negative mode.

Table 4.1: Retention times and mass/ charge ratios (m/z value) of PI-TPα- and COX-2-dependent peaks (Peak 1-3) obtained after HPLC-MS analysis of CMα, CMwt, CMc6 and CMα⁻. Arachidonic acid and several arachidonic acid metabolites served as co-runned standards.

Compound	m/z value	Retention time (min)
Peak 1	309	9.2 / 11.3
Arachidonic acid (AA)	327	14.6
Peak 2	360	15.4
Peak 3	367	14.4 / 15.3
Arachidonoyl ethanolamide (Anandamide, AEA)	370	14.2
Prostaglandin E_2 (PGE_2)	375	7.6
2-arachidonyl glycerol (2-AG)	401	14.2
PGE_2-ethanolamine	418	6.4
PGE_2-glyceryl ester	449	7.2

m/z values of prostaglandins, another group of arachidonic acid metabolites are respectively shorter and higher. Since the three peaks discovered in the present study do not co-elute with any of the standard compounds, identification of these peaks will only be possible when a database of fragments of lipids and lipid metabolites will be established.

Discussion

Both from *in vitro* and *in vivo* studies there is ample evidence to support the hypothesis that the CNS may contain cells, notably astrocytes and other non-neuronal cells, which control neuronal development and maintenance in a PI-TPα-dependent way.

In this study we have characterised the effect of conditioned medium, derived from the C6 astroglioma cell line on the survival of SPIβ cells. We showed that CMc6, like CMα, enhances survival of SPIβ cells when challenged with serum deprivation-induced cell death (Figure 4.1). Since a lipid extract of CMc6 was similarly protective (Figure 4.3), we conclude that CMc6 contains a lipid factor involved in maintaining neuronal integrity and survival.

By using the broad-spectrum GPCR antagonist suramin (data not shown) and SR141716A (rimonabant, Acomplia™), an antagonist of the CB1R, (Figure 4.3) it was shown that the activation of the CB1R, but not the CB2R, is likely to be involved in the survival-enhancing effect of CMc6 and CMα.

Arachidonic acid serves as a precursor for eicosanoid endocannabinoids, the naturally occurring ligands for the CB1R. In agreement with, and similar to, previous studies involving CMα [10], using CMc6 and lipid extracts thereof, we found evidence that endocannabinoid-like lipid factors may contribute to the survival-enhancing effect of CMc6 on SPIβ cells.

In addition, as far as the chemical classification of these compounds is concerned, we showed that arachidonic acid (AA), the major precursor molecule for eicosanoids, as well as the other precursor molecules for eicosanoids EPA, DGLA, and the pre-precursor LA were decreased in CMα and in CMc6, but not in CMwt (Figure 4.4). This indicates an active metabolism of the precursors of eicosanoids in SPIα and C6 astroglioma cells, which may result in high levels of eicosanoids in the medium as previously shown by Schenning et al. for CMα [10].

HPLC-MS analysis of lipid extracts of CMwt, CMα, CMc6, and CMα prepared in the presence of a COX-2 inhibitor ($CM\alpha^{-}$) revealed three PI-TPα- and COX-2-dependent peaks that are exclusively present in CMα and CMc6, (Table 4.1). The m/z ratios and retention times, especially of peak 2 and 3, closely resemble these of the endocannabinoids 2-arachidonyl glycerol and anandamide. Retention times and m/z values of prostaglandins, another group of arachidonic acid-derived eicosanoids are respectively shorter and higher. This information gives another indication for the endocannabinoid-like nature of the survival-enhancing factor(s) in CMc6.

There are several indications that endocannabinoids, which act via CB1R and/or CB2R, do have a protective effect on neuronal cells. Activation of the CB1R during excitotoxicity protects cultured mouse spinal neurons *in vitro* [36]. In addition, cannabinoids have been shown to exhibit a beneficial effect in various mouse models for neurodegenerative diseases, including the models for multiple sclerosis [37], Parkinson's disease [38], Alzheimer's disease [39], Huntington's disease [40] and the motor neuron disease amyotrophic lateral sclerosis [41].

If the nature of the survival-enhancing effect of CMc6 is indeed endocannabinoid-like, this may indicate that these factors might also be responsible, at least to some extent, for the astroglial-mediated protection of neurons *in vivo*. Whether the

production of the survival factors by C6 astroglioma cells is indeed PI-TPα-dependent, has not yet been established in this study. To investigate this hypothesis in more detail, RNA interference-mediated downregulation of PI-TPα expression in C6 astroglioma cells was performed to analyse the protective efficacy of CM derived from these cells. The results of this study are presented in chapter 5.

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Chapter 5:

PI-TPα is involved in the secretion of a bio-active survival factor from C6 astroglioma cells

Hanneke Bunte, Jeroen Vermeulen, Freek L. van Muiswinkel, Karel W.A. Wirtz, and Gerry T. Snoek

Abstract

Reduced expression of the phosphatidylinositol transfer protein α (PI-TPα) has been related to severe (motor) neurodegeneration in several *in vivo* models. *In vitro*, increased expression of this protein has been shown to enhance cell survival. In addition, conditioned medium of cells overexpressing PI-TPα increased the survival of serum-deprived primary motor neurons (chapter 2). As PI-TPα expression was found to be highest in the central nervous system and PI-TPα is abundantly expressed in astrocytes, it is hypothesised that the previously described protective effect of conditioned medium of C6 astroglioma cells (CMc6) might be correlated to PI-TPα expression. To investigate this hypothesis, in the present study RNA interference techniques were used to reduce the PI-TPα protein expression level in C6 astroglioma cells to 10% of its original level. Compared to normal CMc6 the survival-enhancing effect of CM derived from these PI-TPα-depleted cells (CM16) appeared to be reduced when tested on the apoptosis-sensitive SPIβ cells and the motor neuron cell line NSC-34 grown in the absence of serum. In addition, CMc6 was able to reduce H_2O_2-induced oxidative stress in NSC-34 cells as measured with the oxidation-sensitive probe dichlorofluorescein. This protective effect was diminished when these cells were incubated with CM16. From these data it is concluded that conditioned medium from C6 astroglioma cells contains survival-enhancing lipid factors, the production of which is dependent, at least in part, on PI-TPα protein expression. Further investigation on the identification of the survival factor(s) involved is required before they can be applied for therapeutic use.

Introduction

The lipid transfer protein phosphatidylinositol transfer protein α (PI-TPα), which is capable of transferring phosphatidylinositol (PI) and phosphatidyl choline (PC) between membranes *in vitro*, is highly conserved among species [1] and is expressed most prominently in the CNS [2, 3].

In vivo studies showed that a reduced level [4] or total knock-out [5] of PI-TPα protein expression resulted in severe degeneration of the motor neuron system and early death in mice. In addition, reduced PI-TPα expression in zebrafish embryos also resulted in neurodegeneration in the spinal cord and a defect in neurite extension in remaining neurons [6]. Null mutations in RdgBα, a membrane protein with a PI-TP-like domain, result in light-enhanced degeneration of the photoreceptor cells in the retina of *drosophila* [7].

On the other hand, it was found that NIH3T3 mouse fibroblasts overexpressing PI-TPα (SPIα cells) showed increased resistance against induced apoptosis [3, 8]. In addition, these cells produce and secrete survival-enhancing factors. Serum-free medium conditioned by SPIα cells (CMα) was able to protect wild type NIH3T3 cells against apoptosis induced by UV radiation, serum deprivation, or TNFα treatment in a paracrine manner [3]. Interestingly, we described previously that CMα was also able to protect primary motor neuron cultures from serum deprivation-induced cell death [2].

Analysis of SPIα cells revealed that overexpression of PI-TPα resulted in the enhanced production of arachidonic acid, possibly derived from PI. In addition, it was found that cyclooxygenase-2 (COX-2) protein expression was increased [8, 9]. The anti-apoptotic activity of CMα is probably attributable to the secretion and action of a COX-2-dependent arachidonic acid metabolite, since the production of the bio-active factor was inhibited in the presence of a COX-2 inhibitor [8]. Furthermore, it was shown that the bio-active factor acts via a G-protein coupled receptor (GPCR), presumably the cannabinoid-1-receptor (CB1R) [2, 8].

These data indicate that the PI-TPα protein level in the CNS may be crucial in the production of a neuronal survival-enhancing factor that may act, either in an autocrine or paracrine fashion, via a CB1-like receptor.

Analysis of different cell types derived from the central nervous system (CNS) revealed that the PI-TPα level in neuronal cell lines was relatively low [3] (chapter

3). PI-TPα expression in the supporting astroglial cells, however, was found to be much higher and even increased compared to the level in SPIα cells. This led to the hypothesis that astrocytes may secrete a PI-TPα-dependent bio-active factor that is implicated in neuronal maintenance in the CNS. Chapter 4 shows that conditioned medium of C6 astroglioma cells (a well-established astrocyte cell line [10]), is indeed able to protect mouse fibroblasts against serum deprivation-induced cell death. The survival-enhancing activity of this medium was reduced when co-incubated with an antagonist of the CB1R. HPLC/MS analysis of the conditioned medium of C6 astroglioma cells, so-called CMc6, revealed that the survival-enhancing factor may be an endocannabinoid-like compound. These data indicate that the bio-active lipid-related factors produced and secreted by C6 astrocytes are likely to be of the same nature as produced by SPIα cells. However, the involvement of PI-TPα in the survival-enhancing activity of CMc6 has not been unequivocally demonstrated yet.

Therefore, RNA interference techniques were used to reduce PI-TPα protein expression in C6 astroglioma cells. To analyse whether the survival-enhancing effect was altered by this reduction in PI-TPα expression, the conditioned medium of these cells was incubated with the apoptosis-sensitive fibroblast SPIβ cell line [3] and the murine motor neuron cell line NSC-34 [11] under the condition of serum deprivation-induced cell death. Furthermore, since serum deprivation induces the production of oxidants and subsequent oxidative stress [12, 13] it was investigated whether CMc6 was able to protect NSC-34 cells against the consequences of oxidative stress, a phenomenon also often observed during neurodegenerative disorders [14], and whether this was PI-TPα-dependent.

Materials and methods

Chemicals

Culture media and sera (Gibco), lipofectamine™ 2000 (Invitrogen), lipofectamine™ RNAiMAX (Invitrogen), Short interfering double-stranded RNA oligomers (Ambion), PARP antibody (Santa Cruz), Cleaved caspase-3 antibody (Cell Sigaling Technology), monoclonal β-actin antibody (Sigma), ethyl acetate (Merck), formic acid (Merck), ethanol absolute (merck), alamarBlue™ (BioSource International, Inc.), hydrogen peroxide 35% (Merck), 6-carboxy-2',7'-

dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (acetylated H_2DCF) (invitrogen molecular probes C-2938), antioxidant assay kit (Sigma), goat-anti-rabbit horseradish peroxidase antibody (Biorad), goat-anti-mouse horseradish peroxidase antibody (Biorad), Bradford assay (Biorad) and enhanced chemilumincence detection reagents (Amersham Biosciences) were obtained from the suppliers indicated.

Cell culture

NIH3T3 cells overexpressing PI-TPα (SPIα cells) and PI-TPβ (SPIβ cells) were generated as described previously [9, 15]. NIH3T3 cells, SPIα and SPIβ cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose and Glutamax, supplemented with newborn calf serum (NCS; 10%) and penicillin and streptomycin in a humidified atmosphere at 37°C, and 5% CO_2. Cells were split once (SPIβ) or twice (NIH3T3 and SPIα) a week. SPIα and SPIβ cells were grown in G418 (geneticin; 400 μg/ml) to prevent disposal of the transfected vector. During experiments G418 was excluded from the medium.

The motor neuron hybrid cell line NSC-34 (neuroblastoma x mouse spinal cord) [11] was grown in DMEM containing high glucose, pyruvate and glutamax supplied with 10% foetal bovine serum (FBS; heat-inactivated, EU approved) and penicillin and streptomycin in a humidified atmosphere at 37°C, and 5% CO_2. To improve cell attachment, cells were grown in flasks and wells with a modified polystyrene surface (CELL BIND, Costar-Corning). Cells were split approximately twice a week until passage 25-30.

C6 astroglioma cell cultures [10] were grown in Ham's F10 medium containing glutamax, supplemented with 10% FBS and penicillin and streptomycin in a humidified atmosphere at 37°C, and 5% CO_2. Cells were split twice a week upon confluency. Cells were used until passage 30-35.

Selection of RNAi constructs to obtain downregulation of PI-TPα in C6 astroglioma cells

RNAi constructs were selected using the siRNA selection program from the Whitehead Institute for Biomedical Research [16]. The open reading frame (ORF) mRNA sequence of rat PI-TPα obtained from [17] was used as input material. The

siRNA patterns N4AN6TN2HN5WN2 (Protocol Reynolds et al) and AAN21 (Tuschl protocol) were used for selection. The five chosen constructs were selected on thermodynamic criteria, a negative blast against the rat genome, including PI-TPβ, and the absence of internal hairpins. In addition, it was required that the constructs were equally distributed along the sequence. The sequence of one of the constructs (i.e. construct 8, note Figure 5.1) was scrambled to create a negative control.

RNAi assay to downregulate PI-TPα in C6 astroglioma cells

To test which construct(s) resulted in optimal downregulation of PI-TPα, C6 astroglioma cells were plated in wells of a 12-wells plate (4 cm^2) in Ham's F10 medium containing glutamax, supplemented with 10% FBS, without antibiotics ($F10^+$ medium) at a cell density of $2.5x10^5$ cells per well. After 24 h medium was replaced with fresh $F10^+$ medium (volume per well: 800 μl), and a mix of lipofectamine and construct was added. This mixture (200 μl per well) was prepared by adding 6 μl lipofectamine™ to 100 μl Ham's F10 medium containing glutamax without serum ($F10^-$ medium) in an eppendorf tube. After five minutes of incubation at RT, this mixture was added to 100 μl $F10^-$ medium containing 40 pmol of RNAi construct. After an additional 25 minutes of incubation, this lipofectamine-construct mix was added to the cells (final volume: 1 ml). After 72 h of incubation, the cells were washed twice with PBS and stored at -20°C until further processing. To investigate the optimal amount of lipofectamine, 40 pmol of construct 16 was added to increasing amounts of lipofectamine.

Preparation of conditioned media (CM)

CM of NIH3T3 and SPIα cells was prepared as described previously [8]. Briefly, cell cultures of either wild type NIH3T3 mouse fibroblasts or SPIα cells were grown to 80-90% confluence in DMEM containing 10% newborn calf serum. After washing twice with phosphate buffered saline (PBS; pH 7.4), the medium was replaced by serum free DMEM without phenol red containing glutamax and 0.1% BSA (DBB). After 24 h, the medium was collected and centrifuged (5 min at 500g) to yield either CMα or CMwt (from SPIα or wild type NIH3T3 cells, respectively).

To obtain CM from C6 astroglioma cells, cells were plated in a 10-cm dish (55 cm^2) with a cell density of $3.4x10^6$ cells per dish. After 4 days (24+72 h) the cell culture was confluent and was washed twice with PBS. Medium was replaced with DBB and after 24 h (24+96 h in total), the resulting CM was collected and centrifuged (5 minutes at 500 g) to yield CMc6. All conditioned media were stored at 4°C until further use.

SPIβ cells were incubated with CM derived from an identical cell surface (i.e. 1 cm^2 of SPIβ cells was incubated with CM derived from 1 cm^2 of cells).

Preparation of CM from C6 astroglioma cells with downregulated expression of PI-TPα

C6 astroglioma cells were plated in 10-cm dishes (55 cm^2) at a cell density of $3.4x10^6$ cells per dish in $F10^+$ medium. After 24 h the medium was replaced by 10 ml fresh $F10^+$ medium and the lipofectamine-construct mixture was prepared and added as described above (55 μl lipofectamine RNAiMAX in 1.4 ml $F10^-$ medium and 550 pmol of construct 16 or scr in 1.4 ml $F10^-$ medium). After 72 hours of incubation, cells were washed twice with PBS and medium was replaced by 5 ml of DBB. After an additional 24 hours, the medium was collected and centrifuged (5 min; 500 g) to obtain CM16 or CMscr, respectively. Cells were washed twice with ice-cold PBS and stored at -20°C to determine PI-TPα and β levels after CM preparation. CMc6, prepared as described above was used as a control.

Preparation of lipid extracts (LEs)

Lipid extracts (LEs), containing arachidonic acid metabolites, were obtained from DBB, CMc6, CMα, and CMwt as described previously [18]. In short, upon addition of 30 μl of 12 M formic acid to one ml of CM the mixture was extracted with two 3 ml volumes of ethyl acetate. The combined extracts were then evaporated under nitrogen before the residues were dissolved in ethanol to yield LEdbb, LEc6, LEα, and LEwt, respectively, which were diluted in DBB to appropriate concentrations (an extract of x ml CM was rediluted in x ml DBB) was and immediately used after preparation. During the experiments ethanol concentrations never exceeded 0.2%.

Analysis of PI-TPα and β expression in C6 astrocytes treated with RNAi constructs

For the determination of PI-TPα and β expression levels in C6 astroglioma cells treated with RNAi constructs cells were lysed in ice-cold lysisbuffer containing 10 mM Tris (pH non-adjusted) and 0.1% Nonidet P-40 in de-mineralised water. After centrifugation for 10 min at 20,000 g (4°C), the protein concentration in the samples was measured using a Bradford assay [19]. 30 μg Aliquots of total protein were prepared in 5x Laemmli sample buffer [20]. Proteins were resolved on a 10 % SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. 10, 20, 30 and 40 ng of purified PI-TPα was loaded to use as a calibration curve. Subsequently blots were cut to either separate PI-TPα or β (~35 kD) and β-actin (43 kD). After blocking in non-fat milk powder for 1 h, membranes were incubated with an affinity-purified polyclonal rabbit antibody raised against synthetic peptides representing specific epitopes of PI-TPα [21] or against purified His-tagged PI-TPβ [3] (1:500; 1 h and 1:300, overnight, respectively. After washing, a goat-anti-rabbit (PI-TP) or mouse (β-actin) antibody conjugated to horseradish peroxidase (1:5000) was used as a secondary antibody (1 h). Membranes were washed and developed with enhanced chemiluminiscence detection reagents according to the manufacturer's instructions. Quantification of bands on film was performed by scanning with a Bio-Rad GS 700 imaging densitometer equipped with integrating software.

Induction of apoptosis in SPIβ cells by serum deprivation

SPIβ cells were grown until 90-95% confluent in 12- or 6-wells plates in normal growth medium without G418. Cells were washed with PBS twice to remove remaining serum. CM or a LE was added at the appropriate concentration (i.e. 1 cm^2 of SPIβ cells was incubated with CM or LE derived from 1 cm^2 of cells, dilution occurred in DBB). DBB served as a control. After 8 h of incubation cells were washed twice with ice-cold PBS. Medium and PBS were collected and centrifuged to collect floating cells. Pellets were washed with ice-cold PBS. Cells and cell pellets were stored at -20°C until further processing.

Analysis of PARP and cleaved caspase-3 in treated SPIβ cells

Frozen SPIβ cells and cell pellets obtained after induction of apoptosis by serum deprivation were lysed on ice in ice-cold lysisbuffer and centrifuged for 10 min at 20,000 g (4°C). After determining the protein concentration using a Bradford assay [19], 40 and 60 μg aliquots of total protein were prepared in 5x Laemmli sample buffer [20] for the analysis of poly(ADP-ribose) polymerase-1 (PARP) and cleaved caspase-3, respectively.

Proteins were resolved on a 10% SDS-polyacrylamide gel in the case of PARP analysis or a 15% gel in the case of cleaved caspase-3 analysis and transferred to a nitrocellulose membrane. Subsequently blots were cut to either separate (cleaved) PARP (85/116 kD) and β-actin (43 kD) or cleaved caspase-3 (16 kD) and β-actin. After blocking in non-fat milk powder (1 h), membranes were incubated with anti-rabbit PARP antibodies (1:1000), anti-rabbit caspase-3 antibodies (1:000), or anti-mouse β-actin antibodies (1:2000) in diluted non-fat milk powder overnight. After washing, a goat-anti-rabbit or mouse antibody conjugated to horseradish peroxidase (1:5000) was used as a secondary antibody (1 h). Blots were washed and developed with enhanced chemilumincence detection reagents according to the manufacturer's instructions. Quantification of the bands was performed by scanning the film with a Bio-Rad GS 700 imaging densitometer equipped with integrating software. Experiments were performed in duplicate.

Induction and analysis of apoptosis in NSC-34 cells by serum deprivation

NSC-34 cells were plated at a cell density of 1.0×10^4 cells per well (0.32 cm^2) of a 96 wells plate (CELL BIND) in normal growth medium. After 24 hours, cells were washed once carefully with PBS and subsequently incubated with CM (final volume per well: 100 μl). DBB served as a control. After 48 hours, per well 10 μl of alamarBlue™ was added and cells were incubated at 37°C for 2 hours. AlamarBlue™ can be used to assess cell viability and, according to the manufacturer, shows comparable results with an MTT assay. The advantage of alamarBlue™ is that no washing steps are required, which is favourable when using cells that are not well attached like NSC-34 cells. AlamarBlue™ fluorescence was measured using a fluorimeter (FLUOstar OPTIMA, BMG Labtechnologies) with excitation and emission filters of 540 and 590 nm, respectively. Experiments were performed in triplicate.

Detection of hydrogen peroxide-induced oxidative stress in NSC-34 cells

NSC-34 cells were plated at a cell density of $1.5x10^4$ cells per well (0.32 cm^2) of a 96 wells plate (CELL BIND) in normal growth medium. After 24 hours, cells were washed once carefully with PBS and subsequently incubated with 10 μM 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (acetylated-H_2DCF) in DBB (without phenol red) for 30 min. The DCF mixture was removed and 300 μM hydrogen peroxide (H_2O_2) in DBB or CM was added to the cells (100 μl total). DBB without H_2O_2 served as a control. Immediately the fluorescence of DCF (a measure for overall oxidation in the cell) was monitored in a time-resolved manner using a fluorimeter (FLUOstar OPTIMA, BMG Labtechnologies) during the following 2 hours. Excitation and emission filters used were 485 and 520 nm respectively. Starting values were subtracted from obtained values after 2 hours to acquire the increase in oxidation during 2 hours. Experiments were performed at least in triplicate.

Detection of antioxidant activity in DBB or CM

The presence of antioxidants in the conditioned media was measured using an antioxidant assay kit. The mechanism of action of the kit is based on the formation of a ferryl myoglobin radical from myoglobin and H_2O_2, which oxidizes the ABTS (2,2'-azino-bis(3-ethylbenzthiazo-line-6-sulfonic acid) to produce the radical cation $ABTS^+$, a soluble green colour chromogen, which absorption can be determined at 405 nm. In the presence of antioxidants the radical cation, and thus the colour, is produced to a lesser extent, proportionally dependent on the activity of the antioxidant. Trolox™, a water-soluble vitamin E analogue, was used as a standard, or control antioxidant. The assay was performed according to the manufacturer's instructions using 10 μl of CM per well. Two independent experiments were performed in duplicate.

Results

Optimalisation of PI-TPα downregulation in C6 astroglioma cells

In order to downregulate PI-TPα protein expression in C6 astroglioma cells, different double stranded RNA constructs were generated targeted only at the mRNA ORF of this protein. The sense strands of the six RNA constructs that were selected, including the scrambled construct that was used as a negative control in all experiments, are shown in Figure 5.1A. As indicated, the constructs

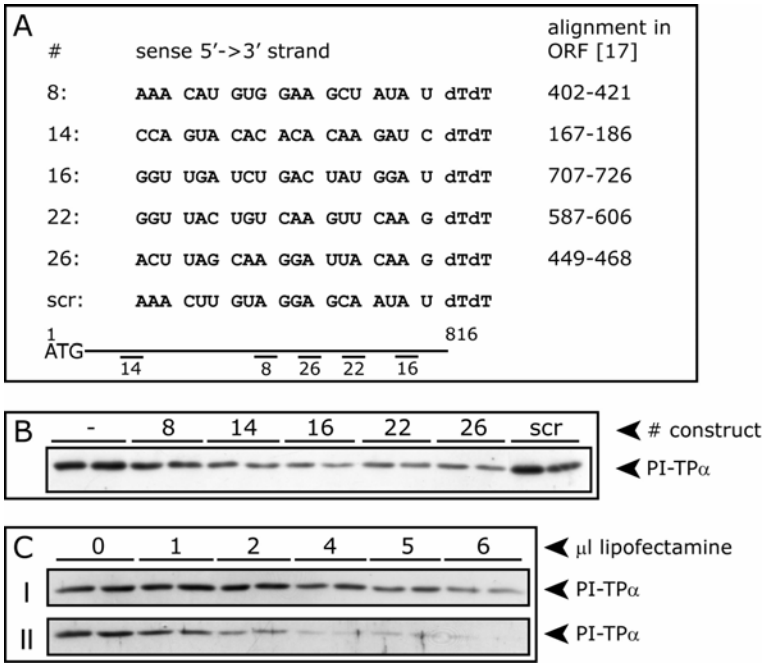

Figure 5.1: Downregulation of PI-TPα with selected double stranded RNA constructs. Panel **A** shows the sense strand of the selected RNA constructs and the alignment in the mRNA open reading frame (ORF) of the protein as described previously [17]. The scrambled (scr) construct is a copy of construct 8 but with 4 nucleotides altered. This construct was used as a negative control. Underneath this, a cartoon of the mRNA sequence of PI-TPα is visualised, showing that the constructs are equally distributed along he sequence. Panel **B** shows a Western blot with PI-TPα protein levels in C6 astroglioma cells 72 hours after addition of the mentioned constructs. Panel **C** shows PI-TPα levels in the same cell line after 72 hours of incubation with construct 16 and different aliquots of lipofectamine 2000 (blot I) or lipofectamine RNAiMAX (blot II). Loading control was performed with a Bradford protein determination assay and Ponceau-S staining after the blotting procedure. Experiments were carried out in 12-wells plates (seeding area: 4 cm^2 per well).

are equally distributed along the mRNA sequence of PI-TPα. The efficiency of downregulation of the different constructs with lipofectamine 2000, the standard lipofectamine agent, in C6 astroglioma cells was determined by Western blot analysis. Figure 5.1B shows that after 72 hours, construct 16 shows the most pronounced decrease in PI-TPα protein expression. As expected, the scrambled construct showed no significant decrease in PI-TPα expression.

During this experiment, it was observed that the lipofectamine 2000 concentrations indicated by the manufacturer were not sufficient for (optimal) downregulation in C6 astroglioma cells. Therefore, a concentration range of different amounts of lipofectamine was incubated with construct 16 to determine the amount of lipofectamine that resulted in optimal downregulation. Western blot I in Figure 5.1C shows that marked downregulation is obtained when the cells were incubated with five to six microliter of lipofectamine 2000 per well (4 cm^2). This is 2.5 to three times as much as the standard amount of lipofectamine indicated by the manufacturer.

In addition, it was observed that incubation of the cells with increased amounts of lipofectamine 2000 gave rise to cytotoxicity. Therefore, another lipofectamine, RNAiMAX, a transfection reagent specially designed and manufactured for delivery of double stranded RNA, was tested for its cytotoxic effect. Whereas the transfection efficiency of RNAiMAX was increased compared to lipofectamin (Figure 5.1C, Western blot II), its cytoxicity was substantially reduced as under these latter conditions a confluent cell layer was attained. Using a calibration curve of known PI-TPα concentrations, it could be calculated that when 4 μl of lipofectamine RNAiMAX was used per well (4 cm^2), PI-TPα expression was downregulated by 85-90 percent after 72 hours compared to 30 percent when the cells were incubated with lipofectamine 2000. Higher amounts of RNAiMAX did not result in a more pronounced downregulation. Because of the above results, further RNAi experiments were carried out using construct 16 and lipofectamine RNAiMAX.

The effect of PI-TPα downregulation on C6 astroglioma cells

As mentioned in chapter 4, preparation of an active survival-enhancing conditioned medium of C6 astroglioma cells (CMc6) requires a confluent cell layer. 96 Hours after seeding, this confluent cell layer was attained. When the cells were

incubated with lipofectamine RNAiMAX and construct 16 or the scrambled construct confluency was again attained after 96 hours (i.e. 24 hours after seeding, the RNAi procedure was started, and 72 hours thereafter a confluent cell layer was attained).

However if, as a control, cells were incubated with lipofectamine without any construct, a confluent cell layer was not reached within 96 hours because of cytotoxicity, as described previously [22]. Therefore, the negative control used was CMc6 grown with neither construct nor lipofectamine. Figure 5.2 shows that under all conditions a confluent cell layer was attained at 96 hours after seeding.

Conditioned medium of C6 astroglioma cells pre-incubated with the different constructs (i.e. construct 16 and scr, yielding CM16 and CMscr respectively) was prepared between 72 and 96 hours after addition of the constructs (96 and 120 hours after seeding). CMscr was used as a control to test for effects as a result of the transfection procedure.

Figure 5.3 shows Western blots with the level of PI-TPα in the cells after the preparation of the conditioned medium (i.e. 24+72+24 hours after seeding). The black bars in the graph indicate that PI-TPα expression in cells incubated with construct 16 decreased to 13 ± 3.1 percent of the original level (100 ± 5.8%). This is lower compared to the level of PI-TPα in wild type NIH3T3 cells (data not shown, chapter 3). PI-TPα expression in cells incubated with the scrambled construct was not significantly decreased (98 ± 13 %).

A negative blast against the whole rat genome predicted that the RNAi procedure

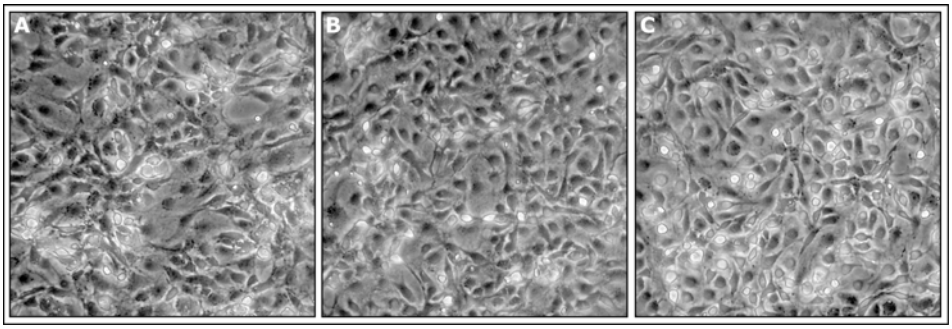

Figure 5.2: A confluent cell layer is attained 72 hours after the start of the RNAi procedure. 24 Hours after seeding culture medium was refreshed and the cells were incubated with or without a prepared mixture of RNAi construct and lipofectamine RNAiMAX. In panel **A** no mixture was added. In panel **B** and **C** cells were incubated with construct 16 and the scrambled construct, respectively. Images were taken 72 hours after addition of the construct/ lipofectamine mixture.

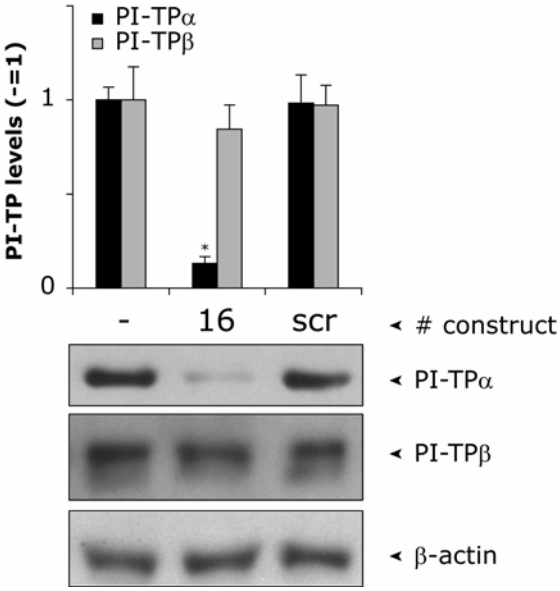

Figure 5.3:
Optimal downregulation of PI-TPα levels in C6 astroglioma cells does not affect PI-TPβ levels. Cells were incubated with no construct (-), construct 16 (16), or a, negative, scrambled construct (scr). After 72 hours, cells were washed and incubated with DBB medium for 24 hours to compose conditioned medium. Medium was collected and cells were washed. PI-TPα and PI-TPβ levels were determined in the cell lysates to analyse downregulation. The shown Western blots demonstrate representative results of four independent experiments. Bars in the graph show averages ± SEM of four independent experiments. * $p < 0.001$ vs. levels in – and scr.

would have no effect on PI-TPβ expression. This was confirmed by Western blot analysis, using specific anti PI-TPβ antibodies, shown in Figure 5.3 (grey bars). During preparation of the conditioned medium, the confluent cell layer was maintained and few floating cells were observed in the serum-free medium (DBB) before centrifugation (data not shown).

The effect of CMc6, CM16 and CMscr on induction of apoptosis in SPIβ cells by serum deprivation

To investigate whether the conditioned medium of C6 astroglioma cells in which PI-TPα protein expression was downregulated (CM16) shows altered protective activity compared to CMc6, these conditioned media were tested on apoptosis-sensitive SPIβ cells. As a control, CM of C6 astroglioma cells, incubated with a scrambled construct (CMscr), was used.

SPIβ cells, incubated for 8 hours with CMc6 (A), CM16 (B), or CMscr (C) in the absence of serum are shown in Figure 5.4A-C. From this Figure it can be concluded that CMc6 and CMscr are equally capable of enhancing the survival of

SPIβ cells. From this it can be concluded that the transfection procedure does not interfere with the protective activity of CMc6.

However, when SPIβ cells were incubated with CM16 (Figure 5.4B), an increased number of 'blebbing' structures on the plasma membrane of the cells can be observed, which is a morphological sign of apoptosis [23]. Morphological scoring of blebbing cells revealed that approximately 5-10% of the cells incubated with

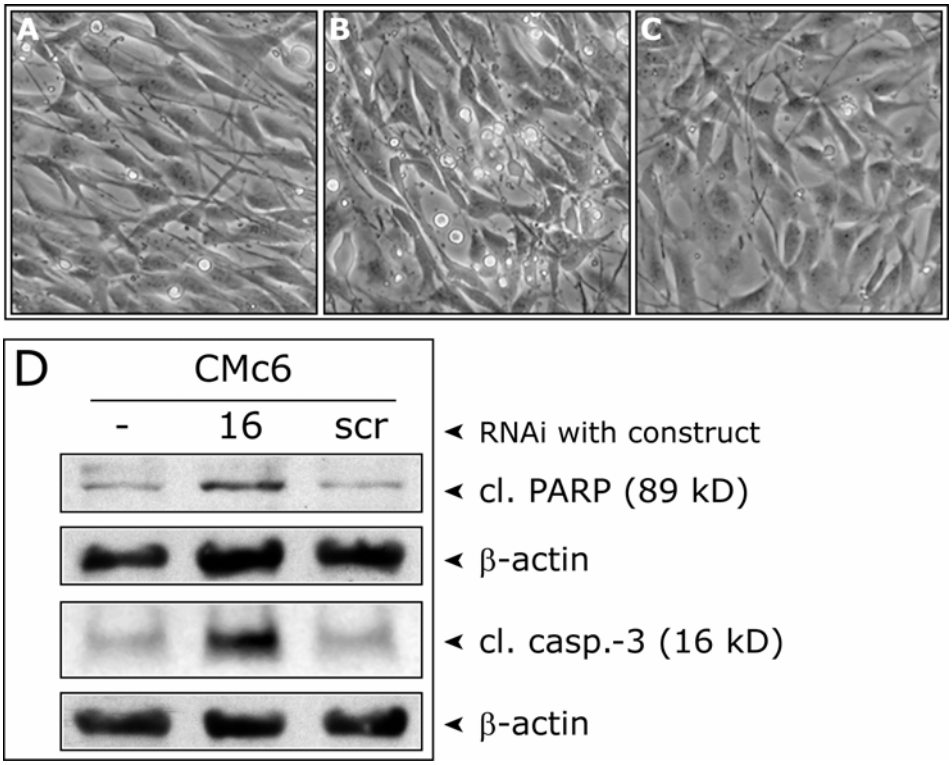

Figure 5.4: RNAi-induced downregulation of PI-TPα in C6 astroglioma cells decreases the protective effect of CMc6. Panel **A-C** show images of SPIβ cells 8 hours after the induction of serum deprivation. Cells were incubated with CMc6 (panel A), CM16 (panel B), or CMscr (panel C) in the absence of serum. Panel **D** shows Western blots of SPIβ cell lysates after 8 hours of serum deprivation in different CM. Blots are immunostained with antibodies against PARP or cleaved caspase-3 to determine the levels of cleaved (cl.) PARP and cleaved caspase-3 and hence, the amount of apoptotic cells, and with β-actin to confirm equal protein loading. Images and Western blot are representative results of six independent experiments.
Abbreviations: CMc6: conditioned medium of C6 astroglioma cells; CM16: CM of C6 astroglioma cells in which PI-TPα is downregulated; CMscr: CM of C6 astroglioma cells incubated with a scrambled RNAi construct that served as a negative control; PARP: poly(ADP-ribose) polymerase-1.

CMc6 are in an apoptotic state, as compared to 2-5% when cells were incubated with CMscr. When SPIβ cells were incubated with CM16, the percentage of blebbing cells increased to 20-25%.
This morphological observation is biochemically verified by detecting caspase-3 and poly(ADP-ribose) polymerase-1 (PARP) cleavage in cell lysates of SPIβ cells 8 hours after incubation with the different conditioned media. Upon induction of apoptosis, pro-caspase-3 is cleaved, which results in the activation of the protease activity of caspase-3. A substrate of active, cleaved, caspase-3 is PARP (113 kD), which is cleaved into two fragments (89 and 24 kD) [24]. Figure 5.4D shows that, upon incubation with CM16 in the absence of serum, the levels of both cleaved caspase-3 and cleaved PARP in SPIβ cells are increased compared to the levels in cells incubated with CMc6 or CMscr. Hence, the caspase-3/actin ratio increases three-fold when cells were incubated with CM16 compared to CMc6 or CMscr. The PARP/actin ratio in cells incubated with CM16 increases approximately five-fold compared to CMc6.
The increase in caspase-3 and PARP cleavage as well as the morphological signs of apoptosis in SPIβ cells incubated with CM16, indicate that the survival-enhancing effect of this medium is decreased compared to CMc6.

The effect of a lipid extract of CMc6, CM16 and CMscr on induction of apoptosis in SPIβ cells by serum deprivation

As described in chapter 4, the survival-enhancing activity of CMc6 is preserved in a lipid extract containing arachidonic acid metabolites. To determine whether the reduced survival-enhancing activity of CM16 may be due to an altered arachidonic acid metabolism, a lipid extract of this medium was prepared. The lipid extracts were diluted in DBB medium and incubated with SPIβ cells for 8 hours in the absence of serum. Figure 5.5, panels A-C, show images of SPIβ cells 8 hours after incubation with a lipid extract from CMc6 (LEc6; panel A), CM16 (LE16; panel B), or CMscr (LEscr; panel C).
Compared to LEc6, a clear increase of blebbing cells can be observed when SPIβ cells were incubated with the LE16. This morphological observation was supported by the observed increase of PARP cleavage in SPIβ cell lysates after incubation with a lipid extract of CM16, as shown by the Western blot in Figure 5.5D. The PARP/actin ratio increased two-fold if cells were incubated with LE16

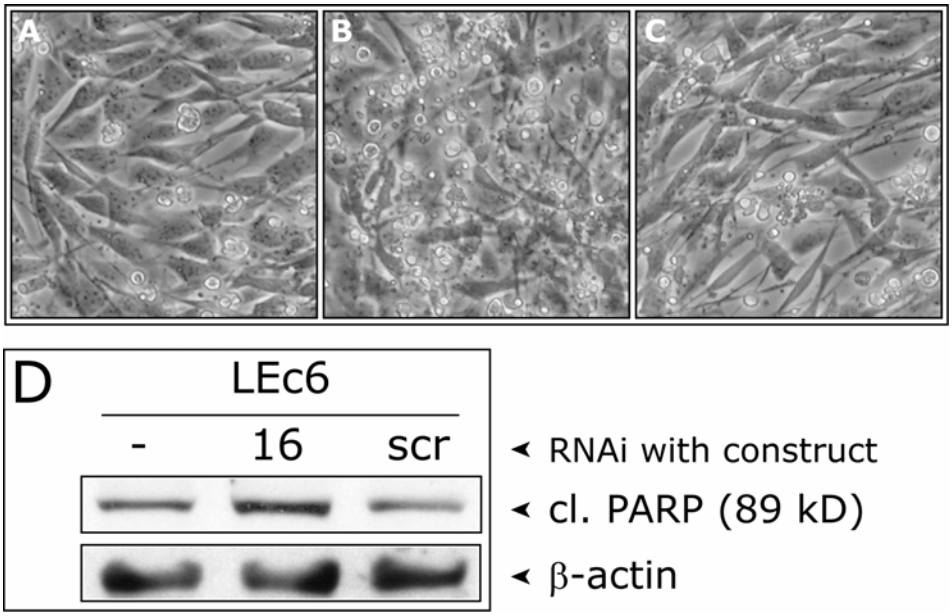

Figure 5.5: RNAi-induced downregulation of PI-TPα in C6 astroglioma cells decreases the protective effect of a lipid extract of the conditioned medium these cells. Panel **A-C** show images of SPIβ cells 8 hours after the incubation with a lipid extract of CMc6 (panel A), CM16 (panel B), or CMscr (pancel C) in the absence of serum. Panel **D** shows a Western blot of SPIβ cell lysates after 8 hours of incubation with the different lipid extracts. Blots are immunostained with antibodies against PARP to determine the levels of cleaved (cl.) PARP and hence, the amount of apoptotic cells, and with β-actin to confirm equal protein loading. Images and Western blot are representative results of three independent experiments.
Abbreviations: CMc6: conditioned medium of C6 astroglioma cells (no pre-incubation with a RNAi construct (- in panel D)); CM16: CM of C6 astroglioma cells in which PI-TPα is downregulated with construct 16; CMscr: CM of C6 astroglioma cells pre-incubated with a scrambled construct that served as a negative control; PARP: poly(ADP-ribose) polymerase-1.

compared with LEc6 or LEscr. No changes in ratios were observed between CMc6 and CMscr. These data indicate that the protective activity of CMc6 may be due to the presence of PI-TPα-dependent arachidonic acid metabolites in the lipid extract.

The effect of CMc6, CM16 and CMscr on induction of apoptosis in NSC-34 cells by serum deprivation

Since downregulation of PI-TPα results in severe motor neuron degeneration in mice [4, 5], it was investigated whether CMc6 was able to protect the motor

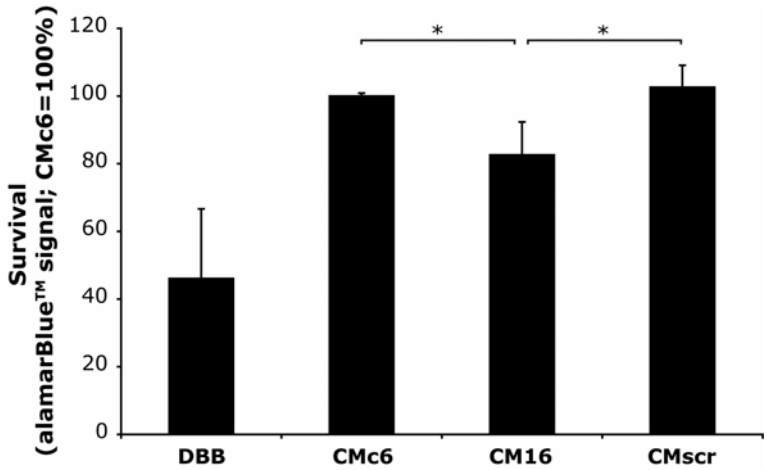

Figure 5.6: RNAi-induced downregulation of PI-TPα in C6 astroglioma cells decreases the protective effect of CMc6 against serum deprivation-induced cell death towards NSC-34 cells. 24 Hours after seeding, NSC-34 cells were incubated with different CM. After 48 hours, alamarBlue™ was added to each well and after two additional hours, fluorescence, as a measure of viable cells, was quantified. The level of fluorescence in wells incubated with CMc6 was set at 100%. Shown values are averages ± SEM of two independent experiments performed in triplicate.
Abbreviations: CMc6: conditioned medium of C6 astroglioma cells; CM16: CM of C6 astroglioma cells in which PI-TPα is downregulated; CMscr: CM of C6 astroglioma cells incubated with a scrambled RNAi construct that served as a negative control.
* $p < 0.01$.

neuron cell line NSC-34 against serum deprivation-induced cell death and whether this protection was PI-TPα-dependent by testing CM16.

In Figure 5.6 the survival of NSC-34 cells after 48 hours of incubation in serum free medium is shown. Cells were incubated with DBB as a negative control. Compared to incubation in DBB, survival of NSC-34 cells in CMc6 is clearly increased (from 48 ± 20% to 100 ± 0.86%). The survival by incubation of the cells with CMscr is not significantly changed. However if cells were incubated with CM16, this resulted in a 33% decrease of the protecting effect of CMc6.

The effect of the CMc6, CM16 and CMscr on the induction of oxidative stress in NSC-34 cells by hydrogen peroxide

Serum deprivation-induced apoptosis is associated with the production of oxidants and subsequent oxidative stress [12, 13]. Therefore we investigated whether CMc6 is able to protect cells against oxidative stress, and if so, whether this protection is

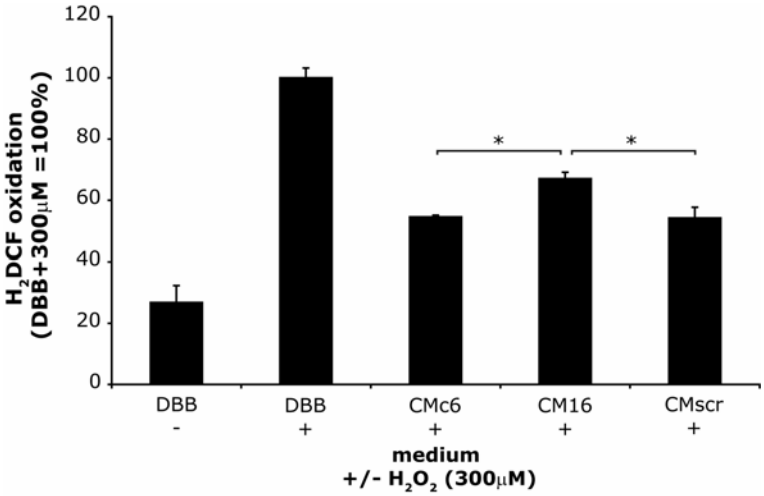

Figure 5.7: RNAi-induced downregulation of PI-TPα in C6 astroglioma cells decreases the protective effect of CMc6 against H_2O_2-induced oxidation towards NSC-34 cells. 24 Hours after seeding, NSC-34 cells were pre-incubated with acetylated H_2DCF and subsequently incubated with different CM with or without 300 μM H_2O_2. Fluorescence of oxidised H_2DCF was determined as a measure of oxidation in the cell. Values represent oxidation levels after 2 hours, subtracted with starting values. Oxidation levels in cells co-incubated with DBB and H_2O_2 were set at 100%. Shown values are averages ± SEM of two independent experiments performed in triplicate.
Abbreviations: H_2O_2: hydrogen peroxide; Acetylated H_2DCF: 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester); CMc6: conditioned medium of C6 astroglioma cells; CM16: CM of C6 astroglioma cells in which PI-TPα is downregulated; CMscr: CM of C6 astroglioma cells incubated with a scrambled RNAi construct that served as a negative control. * $p < 0.01$

PI-TPα-dependent. To this end, NSC-34 cells were loaded with the oxidation-sensitive probe 6-carboxy-2',7'-dichlorodihydro fluorescein diacetate, di(acetoxymethyl ester) (acetylated H_2DCF) and then stressed with 300 μM hydrogen peroxide (H_2O_2) in the presence of different CM. The extent of H_2DCF oxidation after two hours of incubation with CM and H_2O_2 is given in Figure 5.7. H_2DCF oxidation in NSC-34 cells increased slightly in the absence of H_2O_2. This increase was similar for DBB as for the conditioned media tested. In the presence of H_2O_2, H_2DCF oxidation in cells incubated with DBB was increased approximately four times (Figure 5.7). Oxidation was decreased by approximately 60% when H_2O_2 was co-incubated with CMc6 or CMscr, indicating that these media protected the cells against oxidation. When NSC-34 cells were co-incubated with H_2O_2 and CM16, this decrease was only 45%, which is significantly less as observed with CMc6 or CMscr.

Since CM of astrocytes secrete (precursors of) antioxidants [25], the antioxidant status of DBB, CMc6, CM16, and CMscr were assessed in an antioxidant assay. No significant increase in antioxidant activity could be detected (data not shown) in either media, indicating that CMc6 and CMscr trigger an antioxidant-defence in the cell. Since CM16 shows a decrease in this protection, it can be inferred that the protective effect observed in CMc6 might be PI-TPα-dependent.

Discussion

The data presented in this study show that RNAi-induced reduction of PI-TPα protein expression in C6 astroglioma cells reduces the survival-enhancing effect of conditioned medium prepared from these cells on serum-deprived SPIβ cells and NSC-34 cells. In addition, the conditioned medium of PI-TPα-deprived cells was less protective against oxidative stress induced by hydrogen peroxide.

We showed that PI-TPα protein expression in C6 astroglioma cells can be reduced to approximately 10% of its original level using RNA interference techniques. It was previously shown that PI-TPα knock-out embryonic stem cells, can be cultured without increased cell death [26]. We have confirmed that an extensive downregulation of PI-TPα expression does not affect the survival of the ensuing cells.

In mouse fibroblast cells, increased PI-TPα expression is associated with an increased proliferation rate [8]. In our cell system, however, it was shown that cell growth was not affected by PI-TPα downregulation or the RNAi procedure, since a confluent cell layer was reached 72 hours after introduction of active or negative RNAi constructs, which was comparable to cell cultures that were not exposed to the RNAi procedure. This lack of effect may be due to proliferation-inducing factors that are present in the foetal serum. In addition, the RNAi-induced downregulation only becomes manifest after incubation with the construct for more than 48 hours, leaving the level of PI-TPα virtually unaltered during the first two days of cell growth.

In chapter 4 it was shown that the conditioned medium of c6 astroglioma cells (CMc6) has survival-enhancing activity resembling that of the conditioned medium of SPIα cells (CMα) in the sense that the activity was still present in a lipid extract and appears to act via the cannabinoid-1 receptor. In addition, it was shown that

both CMα and CMc6 contain low levels of arachidonic acid and other eicosanoid precursor molecules compared to wild type NIH3T3 cells, indicating that eicosanoid metabolism may be high in C6 astroglioma cells as shown in SPIα cells [8]. However, the involvement of PI-TPα in the survival-enhancing activity of CMc6 has not been unequivocally demonstrated yet.

Therefore, in this study conditioned medium was prepared from C6 astroglioma cells in which PI-TPα was downregulated by RNA interference (CM16). We showed that CM16 demonstrates a reduced survival-enhancing effect towards SPIβ cells grown in the absence of serum. This was not due to cytotoxic effects of the RNAi procedure, since CM of control cells treated with a negative (scrambled) RNAi construct (CMscr) remained equally effective, comparable to CMc6. This indicates that the survival-enhancing activity present in CMc6 is, at least in part, correlated to the level of PI-TPα expression. Analysis of the survival-enhancing effect of a lipid extract prepared from the above conditioned media revealed that also the lipid extract of CM16 was less capable of protecting SPIβ cells against serum deprivation-induced cell death.

Compared with CMc6, it can be concluded that the survival-enhancing activity of LEc6 is decreased compared to CMc6. This may reflect the loss of polypeptide growth factors and protein- or peptide-related (precursors of) antioxidants, such as glutathione, known to be present in conditioned medium of astrocytes and to exhibit survival-enhancing activity [25, 27]. In addition, it is known that the activity of the survival-enhancing factors present in LEα quickly decreases in time after extraction, possibly due to oxidation or the loss of a hydrophilic carrier molecule present in CM (Dr. G.T. Snoek, personal communication).

Since arachidonic acid metabolites are present in the lipid extract, the data obtained may indicate that, because the protective activity of LE16 is decreased compared to LEc6 and LEscr, part of the protective activity of CMc6 may be due to the presence of PI-TPα-dependent arachidonic acid metabolites.

Since downregulation of PI-TPα results in severe degeneration of the motor neuron system in mice [4, 5], it was investigated whether CMc6 was also able to enhance the survival of the motor neuron cell line NSC-34 when grown in the absence of serum. Figure 5.6 shows that indeed CMc6 is able to reduce serum deprivation-induced cell death in these cells. Since incubation with CM16 gives a pronounced decrease of cell survival, as compared to CMc6 and CMscr, it is concluded that a high level of PI-TPα expression in C6 astroglioma cells is

correlated with an increased survival-enhancing activity in the medium of these cells, and hence, capable of increased protection of the motor neuron cell line NSC-34.

The fact that the protective effect of CM16 on NSC-34 cells is reduced to a lesser extent than when tested on SPIβ cells, may reflect intrinsic properties of these cells or may be explained by the fact that the conditions used for (i) the induction of apoptosis (i.e. serum deprivation), and/or (ii) the incubation with CM (i.e. time, concentration) may not be similar for both cell types.

Finally, we showed that CMc6 is able to reduce H_2O_2-induced oxidative stress in NSC-34 cells and that this protective effect is decreased when CM16 is co-incubated with this oxidant. Because CMc6 itself did not exhibit anti-oxidant capacity, this may indicate that a PI-TPα-dependent factor is secreted into CMc6, which in turn is capable of increasing the anti-oxidant defence in the cell. Since the two-hour time span in which oxidation was measured is probably too short for *de novo* protein synthesis, the PI-TPα-dependent bio-active factors present in CMc6 may increase the anti-oxidant capacity of the cells, independent of protein synthesis.

Since it is thought that these factors originate from arachidonic acid, and appear to be metabolised by COX-2, it is noteworthy that the COX-2-dependent arachidonic acid metabolite prostaglandin PGE_2 has been demonstrated to reduce oxidation in neurons challenged with lipopolysaccharide [28].

In conclusion, these data indicate that PI-TPα expression in astrocytes may exhibit a neuroprotective role in neurodegenerative disorders. Intriguingly, in line with this hypothesis, PI-TPα-mediated production of survival factors may be part of an endogenous protective response, operative *in vivo*. Thus, it has been demonstrated that in a mouse model for the motor neuron-degenerating disease amyotrophic lateral sclerosis, PI-TPα protein expression has been upregulated approximately 8 times when the mice start to exhibit paralysis, the first clinical symptom of the disease [29]. Maatkamp and co-authors [30] found that the maximum astrocyte proliferation (as shown by GFAP staining) in this mouse model [31] is observed in the same stage of the disease.

These studies indicate that PI-TPα-dependent production of survival factors may exhibit therapeutic potential. However, the identification of the responsible factor(s) requires further investigation before this system can be considered for therapeutic purposes.

Acknowledgements

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Chapter 6:

Summary and general discussion

&

Nederlandse samenvatting

Summary

In this thesis the relationship between phosphatidylinositol transfer protein α (PI-TPα) and neurodegeneration was investigated using available *in vitro* models, previously established in PI-TPα research, and newly developed models involving cell cultures derived from the central nervous system (CNS).

In ***chapter 1*** the different cell types residing in the brain, neurodegeneration, and PI-TPα are introduced. In addition, previous *in vivo* studies relating PI-TPα and neurodegeneration are summarised.

Chapter 2 shows that mouse fibroblast cells overexpressing PI-TPα (SPIα cells) produce and secrete PI-TPα-dependent lipid factors capable of protecting primary rat, spinal cord-derived, motor neuron cultures. Protection was diminished in the presence of suramin, an antagonist of the G-protein coupled receptor family. Previously, using mouse fibroblasts, it was shown that the cannabinoid-1 receptor (CB1R, a GPCR family member) was involved in the protection mechanism. Interestingly, an antagonist of this receptor (SR141617A, Rimonabant, Acomplia™) was toxic to primary motor neuron cultures grown under serum free conditions. Immunocytochemistry studies showed that PI-TPα is expressed in both primary astrocyte and motor neuron cultures.

To investigate whether the high expression level of PI-TPα in the CNS is associated with the cellular production of PI-TPα-dependent survival-enhancing activity and, hence, neuroprotection, several *in vitro* cell models have been developed in ***chapter 3***. Analysis of the PI-TPα level in different CNS-derived primary cell cultures and immortalised cell lines showed that PI-TPα expression is relatively high in astroglial cells (primary cultures as well as the C6 astroglioma cell line). The level of PI-TPα in neuronal cells was significantly lower. Ultimately, on the basis of our data, a new model has been proposed in which the C6 astroglioma cell line serves as 'survival factor-producing cells' and the motor neuron cell line NSC-34 as a 'target cell'. As a first step in this process, an introductory model was introduced with C6 astroglioma cells in combination with the apoptosis-sensitive SPIβ fibroblast cell line as a target cell line.

This latter model was used in ***chapter 4*** to characterise the survival-enhancing effect of the conditioned medium of C6 astroglioma cells (CMc6). It was shown that CMc6 as well as a lipid extract derived thereof, enhanced survival of SPIβ cells under serum-free conditions. This effect was attenuated when CMc6 was co-incubated with the CB1R antagonist rimonabant. Mass spectrometry analysis revealed low levels of arachidonic acid and other eicosanoid precursors in CMc6, indicating an active eicosanoid metabolism in C6 astroglioma cells. In addition, the presence of three PI-TPα- and cyclooxygenase-2-dependent compounds in CMc6 was demonstrated. The nature of these compounds showed some resemblance to endocannabinoids.
In ***chapter 5*** it was investigated whether the survival-enhancing effect of CMc6 is a PI-TPα-dependent phenomenon. Using RNA interference techniques, the PI-TPα expression level in C6 astroglioma cells was downregulated to 10% of its original level. The survival-enhancing effect of the CM derived from these cells was tested on SPIβ cells as well as NSC-34 cells and appeared to be reduced when compared to normal CMc6. In addition, this CM was less effective in protecting NSC-34 cells against H_2O_2-induced oxidative stress.

Taken together, from these data it can be concluded that at least in C6 astroglioma cells, and possibly also in astrocytes, PI-TPα is involved in the production of (a) lipid factor(s) capable of enhancing the survival of an apoptosis-sensitive fibroblast cell line and a motor neuron-like cell line. As indicated above, although the identity of these factors remains largely unknown, as yet these lipid factors may be classified as arachidonic acid-derived endocannabinoids, acting on the CB1R or a CB1-like receptor.

General discussion

PI-TPα function and neurodegeneration

PI-TPα and its homologues (summarised in Table 1.1) have been shown to expressed in all eukaryotic cell types investigated including mammals, yeast, fungi, parasites, amoebae, fish, fly and worm [1]. Among the different species the protein shows a remarkable sequence homology [2]. In addition, evaluation of the expression level of PI-TPα in several mouse [3] and rat [4] tissues and various cell

lines [3] demonstrated that PI-TPα was expressed in all tissues and cell lines investigated with highest expression found in CNS tissue.

In vivo, a reduced PI-TPα expression is associated with neurodegeneration. Studies showed that a reduced level (vibrator mouse) [5] or total knock-out [6] of PI-TPα protein expression resulted, in addition to other symptoms (summarised in Table 1.2), in severe degeneration of the motor neuron system and early death in mice. In addition, reduced PI-TPα expression in zebrafish embryos also resulted in neurodegeneration in the spinal cord and a defect in neurite outgrowth in remaining neurons [7]. Null mutations in RdgBα, a membrane protein with a PI-TP-like domain, result in light-enhanced degeneration of the photoreceptor cells in the retina of *drosophila* [8].

These characteristics indicate that PI-TPα fulfils important functions in cells and organisms, and moreover, may be critically involved in the development and survival of neuronal tissues in particular. However, the precise cellular function of this protein has not yet been revealed.

PI-TPα and cell survival

To investigate the cellular function of PI-TPα, NIH3T3 mouse fibroblasts overexpressing PI-TPα were generated previously. Biochemical analysis of these so-called SPIα cells showed that overexpression of PI-TPα results in the activation of a phospholipase A (PLA) with affinity for PI, resulting in an enhanced production of lysophosphatidylinositol and other degradation products like glycerophosphoinositol, inositol-1-phosphate, inositol-2-phosphate and arachidonic acid as well as arachidonic acid metabolites. In addition, expression of cyclooxygenase-2 (COX-2) was increased [9, 10]. SPIα cells are extremely resistant against UV-induced apoptosis when compared to wild type NIH3T3 cells. Interestingly, conditioned medium from SPIα cells (CMα) was shown to confer protection of SPIβ cells (i.e. NIH3T3 fibroblasts overexpressing PI-TPβ, resulting in a cell line highly sensitive towards apoptosis) against apoptosis induced by UV radiation, serum deprivation, or TNFα treatment [3]. This protection is probably obtained by the secretion and action of a COX-2-derived arachidonic acid metabolite, since production of the bio-active factor was reduced in the presence of a COX-2 inhibitor [10]. Furthermore, it was shown that the bio-active factor

may act via a G-protein coupled receptor (GPCR), presumably the cannabinoid-1-receptor (CB1R) [10].

These data, together with the observation that PI-TPα is abundantly expressed in the CNS [3], has led to the hypothesis that PI-TPα-dependent neuroprotective factors may be implicated in neuronal survival and neurodegeneration. Our data in chapter 2 showed that, similar to SPIβ cells, PI-TPα-dependent lipid factors in the conditioned medium of SPIα cells are capable of protecting primary, spinal cord-derived, motor neuron cultures, thus indicating that these compounds are indeed neuroprotective.

PI-TPα level in the brain

In vitro, a PI-TPα-dependent survival-enhancing effect has been shown to operate in an autocrine as well as a paracrine mode of action [10, 11]. Hence, SPIα cells are extremely resistant towards induced apoptosis when compared to wild type fibroblasts. Besides, the CM of SPIα cells is able to enhance survival of wild type fibroblasts and primary motor neuron cultures. Therefore, in principle, in the CNS a neuron could enhance its own survival in an autocrine fashion provided that these cells express a sufficient level of PI-TPα.

Immunocytochemistry showed that PI-TPα is present in primary neurons [11]. However, Western blot analysis in chapter 3 and [3] showed that the PI-TPα level in neuronal cell lines is relatively low, especially when this level is compared to the level of PI-TPα in astrocytes. Therefore we propose a paracrine mode of action of PI-TPα-dependent survival-enhancing factors, and that glial cells are involved in the production thereof. However, since PI-TPα is expressed in primary motor neurons, it can not be ruled out that a (small) autocrine survival-inducing effect is active in these cells.

Although the involvement of other glial cell types in providing PI-TPα-dependent neuroprotective factors in the CNS can not be excluded at this stage, we hypothesise that astrocytes are primarily responsible for the production and secretion of PI-TPα-dependent survival-enhancing factors, which provide neuroprotection in a paracrine mode of action.

This may be true because the levels of PI-TPα in primary astrocytes as well as C6 astroglioma cells are higher when compared to SPIα cells. Moreover, the

neuroprotective activity of the conditioned medium derived from C6 astroglioma cells was partially dependent on PI-TPα expression. In addition, the astrocyte is the most abundant glial cell type in the brain. Furthermore, it was previously shown that astrocytes (i) are known to express COX-2 and PLA_2 [12, 13], which also appear to be important in the production of the PI-TPα-dependent survival-enhancing factor [9, 10], (ii) are associated with protective activity towards neurons [14-17], and (iii) increase motor neuron survival in motor neuron - astrocyte co-cultures *in vitro* [18]. Finally, (iv) astogliosis (i.e. astrocyte proliferation) has been implicated in the pathophysiology of many neurodegenerative diseases [19].

CMc6 vs. CMα: similarities in survival-enhancing activity

When reviewing the data in chapter 2, 4, and 5 we can conclude that the survival-enhancing activity of CMc6 shows close resemblance with the survival-enhancing activity of CMα towards SPIβ cells as well as neuronal cultures. The survival-enhancing effect of both conditioned media is attenuated in the presence of an antagonist of the CB1R, but not the CB2R. Lipid extracts derived from both conditioned media demonstrate survival-enhancing activity, indicating that a hydrophobic lipid factor may be involved in the activity of CMα and CMc6. Moreover, the survival-enhancing activity of both conditioned media is PI-TPα-dependent as shown by the decreased activity of the CM of wild type NIH3T3 cells (CMwt) and the reduced activity of C6 astroglioma cells with RNAi-induced downregulation of PI-TPα expression (CM16). The same holds for the lipid extracts derived of these conditioned media.

By analysing total lipid extracts of CMα and CMc6 using LC/MS it was found that in both media the levels of arachidonic acid and other eicosanoid precursor molecules were decreased compared to CMwt. This indicates a high level of eicosanoid metabolism in both SPIα cells and C6 astroglioma cells as has been shown for SPIα cells [10]. Using HPLC/MS the PI-TPα- and COX-2-derived compounds present in total lipid extracts of CMα were determined. Three of these compounds were also present in a total lipid extract of CMc6, indicating that these compounds may be candidates for the survival-enhancing effect exhibited by both media. These resembling peaks showed to have hydrophobic properties similar to

arachidonic acid and the arachidonic acid-derived, COX-2-metabolised endocannabinoids 2-arachidonyl glycerol and anandamide, but not to prostaglandins. This indicates that the possible survival factors present in both CMc6 and CMα may be structurally related to endocannabinoids.

From these data it is concluded that both SPIα cells and C6 astroglioma cells exhibit PI-TPα-dependent survival-enhancing activity. The factor(s) involved in this activity may well be of an eicosanoid-derived endocannabinoid nature.

Altered level of PI-TPα *in vivo*: possible causes and effects

The PI-TPα level was found to be altered in two disease mouse models. Thus, Chalimoniuk et al. reported that the PI-TPα level in the hippocampus, but not in the cortex, was increased 20-55% after ischemia and during reperfusion in gerbils [20]. Additionally, expression of PI-TPα has been shown to be upregulated approximately 8 times in a transgenic mouse model of amyotrophic lateral sclerosis (ALS) [21], a fatal, progressive disease in which the motor neuron system degenerates [22]. The mice ubiquitously express human mutant Cu/Zn superoxidedismutase-1 (SOD-1), which is responsible for 2% of the cases in human patients [23]. The increase in PI-TPα level was observed in mice that already showed paralysis as the first clinical sign of the disease (24-28 weeks old) but not in asymptomatic mice (6 to 8 weeks old).

Under these conditions (ischemia and ALS) an increase in astrocyte proliferation is observed [24, 25]. In the used ALS mouse model (generated by Gurney et al. [26]), Maatkamp and co-authors [27] found that a moderate increase in astrocyte proliferation (measured with GFAP staining) starts at week 20-24 and peaks from week 25-29. Motor neuron loss rises from less than 20% in week 20-24 to up to 60% in week 25-29.

Based on the conclusions of this thesis, the above astrocyte proliferation and parallel increase in PI-TPα level might be explained as a defence mechanism of the CNS. Induced neuronal injury may be a signal for the CNS to increase PI-TPα expression, e.g. by astrocyte proliferation. By increasing the production and secretion of the PI-TPα-dependent bio-active factor(s), the CNS might attempt to protect neurons against exogenous- or endogenous-induced degeneration.

The conclusions drawn in this thesis might explain, at least to a certain extent, the neuronal effects observed in the vibrator and PI-TPα$^{-/-}$ mice. The absence of PI-TPα expression in astrocytes of the PI-TPα$^{-/-}$ mice may result in the loss of the PI-TPα-dependent anti-apoptotic factors in the CNS that is required for neuronal development and maintenance. PI-TPα expression in vibrator mice was 80% decreased. This decrease might be just sufficient to produce traces of the factors, increasing the life span of the vibrator mouse from 14 days (life span of the PI-TPα$^{-/-}$ mice) to 40 days.
The fact that these mice show a relatively normal embryonic development and neurite outgrowth may be explained by the earlier raised hypothesis that, during pregnancy, the required PI-TPα-dependent factors are provided via maternal supply [10, 28]. After birth, this supply mechanism is eliminated and the mice start to exhibit the consequences of the lack of production of the PI-TPα-dependent factors. This should possibly affect the whole neuronal system in the CNS.
It could be that motor neurons and neurons in the cerebellum (purkinje cells) are affected first, because of the high vulnerability of these cells. Both cell types belong to the largest cells in the CNS, which consequently results in a high energy demand and a high level of metabolism. As a consequence, this makes these neurons highly susceptible to exogenous changes like the disappearance of a required survival factor. Motor neuron degeneration in the brain stem causes the elimination of the function of vital organs like the lungs. This causes accelerated death of the animal.

Therapeutic potential

The PI-TPα-dependent production of survival factors may have a high therapeutic potential. However, the precise production mechanism and the identification of the responsible factors require further investigation before this system can be applied for therapeutic purposes.
Nevertheless it is tempting to speculate about the effect of increasing the level of PI-TPα in astrocytes in mouse models for neurodegeneration. Techniques to increase PI-TPα expression in the CNS are available. For example, some of these techniques have been applied in the transgenic mouse model for ALS.
To obtain chimerical mice expressing mutant and wild type human SOD-1, Clement et al [29] injected wild type embryonic stem cells into blastocysts overexpressing human mutant SOD-1 or four days after fertilization the authors

mixed wild type morulae cells with morulae expressing human mutant SOD-1. This resulted in mice with varying levels of chimerism. In our formulation the embryonic stem cells or morulae would be cells overexpressing PI-TPα. However, when using this technique, it would be quite a coincidence if these overexpressing cells would differentiate into astrocytes.

Another approach could be the delivery of the PI-TPα gene in a viral vector (i.e. gene therapy). This has already been established using adeno-associated virus particles, including a gene for insulin-like growth factor 1 (IGF-1) which were injected in the muscle of transgenic ALS mice and transferred to the spinal cord via retrograde transport by motor neurons [30]. This, however, resulted in expression of the encoded protein in the motor neurons and not in the astrocytes. The virus particles could also be injected directly into the spinal cord where the particles will transduce neuronal as well as glial cells (reviewed in [31]). By including an astrocyte-specific promoter, PI-TPα will only be expressed in transduced astrocytes. This technique can also be applied to the vibrator or PI-TPα$^{-/-}$ mice to investigate whether the expression of PI-TPα in astrocytes might reduce the CNS-related symptoms.

Besides the beneficial effect of a high level of PI-TPα, the investigations carried out in this thesis also indicate that the CB1R or a CB1-like receptor is involved in the PI-TPα-dependent protection mechanism. In addition it was found that the PI-TPα- and COX-2-dependent compounds found in both CMα and in CMc6 shows characteristics similar to endocannabinoids. In the CNS, activation of the cannabinoid system (i.e. the CB1 and CB2 receptors, the endogenous endocannabinoid ligands that activate them, and the enzymes for the biosynthesis and inactivation of these ligands) has been described as being beneficial in neurogenesis [32-34], the prevention of neurodegeneration [35-37], and, strikingly, liver diseases [38] (note the liver abnormalities in the PI-TPα$^{-/-}$ and vibrator mice).

Van der Stelt et al. [39-41] described that cannabinoids *in vitro* as well as *in vivo* protect neurons against neurodegeneration induced by excitotoxicity, caused by overexcitement of neurons. Abood and co-authors claimed the same outcome for spinal neurons when the CB1R was activated by cannabinoids [42]. Recently, Berghuis et al. [43] identified endocannabinoids as "axon guidance cues" and demonstrated that "endocannabinoid signalling regulates synaptogenesis and target selection *in vivo*". Furthermore, it has been shown in mouse models for different diseases that the cannabinoid system is neuroprotective. Hence, endocannabinoids

have been shown to delay disease progression in the transgenic mouse model for ALS [44]. In addition cannabinoids have shown to act beneficial in models for the neurodegenerative diseases multiple sclerosis [45], and Parkinson's [46], Alzheimer's [47], and Huntington's disease [48].

From these studies and the results obtained in this thesis, it can be concluded that the stimulation of the endocannabinoid system could be of enormous therapeutic importance in developing methods preventing and treating neurodegenerative diseases.

In this concept, therapeutic developments that shut down (part of) the endocannabinoid system must be viewed with great reluctance. From cannabis/marijuana smokers it was known that their habit increased appetite. This increase in appetite was due to the activation of the CB1R by its major psychotropic component, delta(9)-tetrahydrocannabinol [49]. This suggested that the endocannabinoid system is also involved in controlling the energy balance of the body. Since obesity is widely recognised as a serious health problem, that is increasing in prevalence across the world, pharmaceutical companies made a lot of effort to develop a 'diet pill'. The pharmaceutical company Sanofi Aventis developed a drug, called SR141716A (Rimonabant, Acomplia™). Rimonabant selectively blocks the CB1R [50]. Clinical studies have shown that a daily dose of rimonabant produces significant decreases in weight and waist circumference in obese human subjects and improves the lipid profile and glucose control. The frequency of metabolic syndrome also decreased significantly [51]. Since administration of rimonabant does not change the life style of the patient, the drug has to be taken for life. At the moment limited data are available regarding the pharmacokinetics and pharmacodynamics of rimonabant. Adverse reactions have been described, with nausea, dizziness, diarrhea, arthralgia, and back pain being the most common. Psychiatric disorders, including depression and anxiety, were the most common reasons for subjects to withdraw from rimonabant studies. (reviewed in [51]). It is known that the endocannabinoid system plays a role in suicide and depression (reviewed in [52]).

Because of the connection between the administration of rimonabant and the development of psychiatric disorders one must have great reservations about admitting rimonabant on the pharmaceutical market. Moreover, the positive actions of the activation of the endocannabinoid system on neuroprotection and neurogenesis raise important questions to the long-term adverse effects of the administration of rimonabant, especially on administration to obese children. In

this respect it may be relevant that incubation of primary motor neuron cultures with rimonabant in the absence of serum, attenuates the survival of these cells (see chapter 2)
It is to be noted that very recently, an FDA advisory panel of outside experts unanimously recommended that the regulatory agency should not approve this novel weight-loss drug for sale in the United States (press release 18th of June 2007) [53].

Concluding remarks

Although the actual function of PI-TPα, and hence its precise role in neuronal survival and neurodegeneration has not yet been fully elucidated, from the data presented in this thesis the picture emerges that PI-TPα is likely to play an endogenous neuroprotective role in the CNS. Although neurons and other non-neuronal cells can not be excluded, we propose that a high level of PI-TPα expression in astrocytes may be implicated in the production and secretion of PI-TPα-dependent neuroprotective factors *in vivo*. Further in depth *in vitro* and *in vivo* research is required to corroborate this hypothesis and reveal the exact function of PI-TPα in neurodegeneration. If fully elucidated, the production and application of neuroprotective PI-TPα-dependent factors may be an interesting therapeutic target that warrants evaluation in the context of motor neuron diseases and other acute and chronic neurodegenerative diseases.

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Nederlandse Samenvatting

De cel

Elk organisme op aarde is opgebouwd uit één of meerdere cellen (cellula is Latijn voor klein compartiment) die je kunt vergelijken met een kleine fabriek. Meercellige organismen bevatten verschillende soorten cellen met verschillende functies. Bij de mens zijn dat bijvoorbeeld levercellen, niercellen, botcellen enz. Ofschoon verschillende soorten cellen verschillende functies hebben, zijn ze grofweg hetzelfde opgebouwd uit diverse onderdelen ofwel organellen.

In de ***kern*** (de directiekamer) ligt de genetische informatie opgeslagen in de vorm van DNA. Die is voor alle cellen hetzelfde. De gedeelten van het DNA die daadwerkelijk gebruikt worden voor het functioneren van de cel bepalen welke functie een cel heeft. De genetische informatie voor de kleur van je ogen zit dus ook in een levercel, maar wordt daar niet gebruikt. Relevante stukken DNA worden gecopieerd (het heet dan RNA) en naar het ***endoplasmatisch reticulum*** vervoerd. Hier worden de stukjes RNA gelezen als een soort bouwpakket. Uit de verschillende bouwstenen (aminozuren) worden eiwitten gemaakt. In het endoplasmatisch reticulum en daarna in het ***golgi systeem*** worden er aan de eiwitten vaak nog extra onderdelen gehangen, bijvoorbeeld suiker- of fosfaatgroepen.

Eiwitten hebben vele functies in de cel. Zo zijn ze betrokken bij o.a. chemische omzettingen zoals de stofwisseling, de structuur (vorm) van een cel, transport van stoffen in en uit de cel en de communicatie binnen de cel en tussen cellen onderling. De energie die nodig is om de eiwitten te maken, wordt geleverd door de ***mitochondria*** waar energie vrijkomt bij de verbranding van glucose (druivensuiker, de benzine van het menselijk lichaam).

Organellen en cellen worden van hun omgeving gescheiden door een membraan. Dit membraan is een vetachtige, flexibele, dunne wand en bestaat uit lipiden en eiwitten. Lipiden bevatten één of meerdere hydrofobe (waterafstotende) vetzuurstaarten en een hydrofiele (waterminnende) kopgroep. Lipiden zijn een reserve-energiebron voor het lichaam, maar zijn ook belangrijk bij de communicatie in en tussen cellen. Een membraan van alleen lipiden laat heel weinig stoffen door. Cellen hebben stoffen van buiten het membraan nodig om te overleven. Denk bijvoorbeeld aan de aanvoer van glucose voor de energiehuishouding of bouwstoffen voor eiwitten. Ook is het belangrijk dat cellen signalen van andere cellen ontvangen. Ze zijn immers onderdeel van een groter

geheel. Daarom zitten er eiwitten in het membraan verankerd. Deze eiwitten spelen een belangrijke rol bij het transport van moleculen van de ene naar de andere kant van het membraan. Sommige van deze membraaneiwitten hebben een porie (opening). Als de porie open is, kunnen bepaalde moleculen door de porie getransporteerd worden. Echter, niet alle stoffen kunnen/mogen zomaar elke cel in. Hiervoor bestaat een ingenieus systeem. Voor sommige stoffen zijn bijvoorbeeld speciale poriën en sommige stoffen kunnen bepaalde cellen niet in.
Voor het doorgeven van signalen van buitenaf beschikken cellen onder andere over het ligand/receptor systeem. Hierbij scheidt een cel een ***ligand*** (sleutel) uit die past op een ***receptor*** (slot) van de cel aan wie het een signaal wil doorgeven. De receptor is een membraaneiwit, steekt door de membraan heen, en is dus in contact met zowel de buitenkant van de cel (waar de ligand bindt) en de binnenkant van de cel. De sleutel/slot verbinding zorgt voor een kettingreactie in de cel. Als gevolg hiervan vindt er een verandering plaats in de cel. Een cel kan bijvoorbeeld in apoptose gaan (zelfmoord plegen) of zich juist beter wapenen tegen schadelijke invloeden van buitenaf. Het soort verandering hangt af van het soort ligand en receptor. Niet elke cel beschikt over alle soorten receptoren waardoor er een vorm van selectie ontstaat.

Het centraal zenuwstelsel en neurodegeneratie

Dit proefschrift gaat over het ***centraal zenuwstelsel*** (***CZS***, de hersenen en het ruggenmerg samen), één van de meest complexe organen van het lichaam. Het CZS bestaat uit verschillende soorten hersencellen. Het bekendst zijn de zenuwcellen ofwel ***neuronen***. Dit zijn de cellen die er voor zorgen dat men hoort, voelt, ziet, ruikt en proeft, maar voor ook het aansturen van de spieren (o.a. om te bewegen) en het opslaan van gebeurtenissen (geheugen) zijn neuronen verantwoordelijk. Al deze bezigheden zijn gecentraliseerd in verschillende gebieden in de hersenen en worden uitgevoerd door verschillende soorten neuronen. Een neuron in de oogzenuw (sensorisch neuron) heeft heel andere eigenschappen dan (en is moeilijk te vergelijken met) een neuron dat het ruggenmerg verbindt met een skeletspier (***motorisch neuron***). In Hoofdstuk 1, Figuur 1.2 is een motorisch neuron weergegeven.
Om het nog gecompliceerder te maken, zijn neuronen niet de enige cellen in het CZS. Sterker nog, maar 10% van alle cellen in het CZS zijn neuronen. De andere 90% zijn ***glia cellen***. Zoals de naam doet vermoeden (glia is Grieks voor lijm)

dacht men bij het ontdekken van deze cellen (in de tweede helft van de 19e eeuw) dat hun belangrijkste taak was om als een soort cement de neuronen bij elkaar te houden. Pas later bleek dat glia cellen ook noodzakelijk zijn voor het optimaal functioneren van het CZS. Hoe precies, dat wordt nog steeds intensief onderzocht.
De drie belangrijkste soorten glia cellen zijn oligodendrocyten, microglia cellen en astrocyten. Oligodendrocyten zijn het isolatiemateriaal van het CZS. Deze cellen wikkelen zich om de lange uitlopers (axonen) van neuronen om ervoor te zorgen dat de electrische signalen die door het axon lopen, nog sneller gaan en niet kunnen 'overspringen'. De microglia cellen zijn mobiel en verplaatsen zich naar gebieden met dode cellen om ze op te ruimen. Ook spelen zij een belangrijke rol bij ontstekingen. Over de rol van ***astrocyten*** wordt steeds meer bekend. Ze zijn o.a. belangrijk bij de opname van glucose uit het bloed en dus voor de energiehuishouding van neuronen. Bovendien zorgen ze ervoor dat gebruikte boodschapperstoffen die uitgescheiden worden door neuronen om signalen door te geven (neurotransmitters) worden opgeruimd, zodat ze de boodschap niet vaker doorgeven dan noodzakelijk is, wat schadelijk kan zijn. Verder zijn astrocyten betrokken bij het opslaan van informatie (het geheugen) en het zich aanpassen van het CSZ aan nieuwe situaties (leren, plasticiteit). Ook is het bekend dat microglia cellen en astrocyten stoffen (o.a. groeifactoren en anti-oxidanten) uitscheiden die ervoor zorgen dat neuronen beter kunnen overleven.
Figuur 1.6 in Hoofdstuk 1 laat zien hoe neuronen en de verschillende soorten glia cellen zich in het CZS met elkaar verhouden.
Omdat in het CZS verschillende soorten neuronen aanwezig zijn die alle in verschillende mate gevoelig zijn voor in- en externe invloeden, bestaan er veel soorten neurologische aandoeningen. Een voorbeeld zijn de neurodegeneratieve ziekten. Bij deze ziekten zorgt een in- of externe factor (of factoren) ervoor dat een bepaald soort neuronen met één soort functie afsterven (degenereren).
Bij de ziekte van Alzheimer degenereren bijvoorbeeld de neuronen die betrokken zijn bij het korte-termijn geheugen en bij de ziekte amyotrofische lateraal sclerose (ALS) specifiek de motorische neuronen die de skeletspieren aansturen.
De reden voor het afsterven van neuronen bij deze ziekten is vaak niet bekend en hier wordt intensief onderzoek naar gedaan. Wat dit onderzoek complex maakt, is dat de oorzaak van dit soort ziekten vaak multifactoriaal is. Dat wil zeggen dat meerdere, vaak zowel genetische als omgevingsfactoren aan de ziekte ten grondslag kunnen liggen. Het wordt steeds duidelijker dat de rol van glia cellen bij neurodegeneratieve ziekten jarenlang is onderschat. Het blijkt dat glia cellen

enerzijds kunnen bijdragen aan de progressie van de ziekten door bijvoorbeeld het opwekken van ontstekingsreacties. Anderzijds is gebleken dat glia cellen ook in staat zijn neuronen te beschermen tegen degeneratie.

PI-TPα

In dit proefschrift is onderzoek gedaan naar de invloed van het eiwit fosfatidylinositol transport eiwit alfa (in het Engels wordt de afkorting PI-TPα) op neurodegeneratie. ***PI-TPα*** (spreek uit pee-ie-tee-pee alfa) is een eiwit dat betrokken is bij het vervoeren van lipiden door de cel. De lipidstaarten van de lipiden zijn niet oplosbaar in het waterige milieu van de cel waardoor het lipid zich niet zonder hulp door de cel kan verplaatsen. PI-TPα vouwt zich om de lipidstaarten heen en schermt zo het waterafstotende deel van het lipide af waardoor vervoer wel plaats kan vinden.

In muizen komt PI-TPα het meest voor in het CZS (in vaktermen: de ***expressie*** van PI-TPα in het CZS is hoog). Verder is bekend dat een muis die maar 20% van de normale hoeveelheid PI-TPα aanmaakt, door een fout in het DNA, maximaal 40 dagen na de geboorte sterft (een normale muis leeft ongeveer 3 jaar). De reden hiervoor is o.a. het afsterven van de motorische neuronen in het ruggenmerg en de hersenstam en het afsterven van neuronen in de kleine hersenen (cerebellum). Deze laatste soort neuronen zorgt voor de fijne motoriek. Omdat die bij deze muizen verdwijnt, gaan de muizen trillen voor ze iets doen (actie-tremor). Daarom wordt deze muis ook wel de '***vibratormuis***' genoemd.

Met behulp van genetische manipulatie is ook een muis gemaakt die helemaal geen PI-TPα maakt (de ***PI-TPα knock-out muis***). Het stukje DNA dat codeert voor PI-TPα is dan gewist (uitgeknockt), waardoor hier geen RNA meer van gemaakt kan worden en dus ook geen PI-TPα eiwit.

Net als bij de vibratormuis degenereren ook de motorische neuronen en de neuronen in het cerebellum van deze muis. Omdat helemaal geen PI-TPα aanwezig is, gaat dit nog sneller en de muis sterft al binnen 14 dagen na de geboorte.

Hieruit blijkt dat PI-TPα belangrijk is bij de overleving van in ieder geval motorische neuronen en de neuronen in het cerebellum.

Bij de geboorte van beide muissoorten zien ze er net zo uit als normale muizen, wat erop wijst dat de embryonale ontwikkeling normaal is en de degeneratie pas na de geboorte begint.

Om de functie van PI-TPα in meer detail te onderzoeken zijn cellen (geen hersencellen) gemaakt die meer PI-TPα tot expressie brengen dan normaal (***SPIα cellen***). Na onderzoek bleek dat SPIα cellen zich veel beter konden beschermen tegen celdood vergeleken met de normale cellen. Bovendien bleek dat SPIα cellen lipidachtige stoffen uitscheiden die, als je deze aan normale cellen aanbied, ervoor zorgen dat ook deze cellen beter beschermd zijn tegen celdood. Dat betekent dus dat, onder invloed van PI-TPα, beschermende lipidachtige stoffen gemaakt en uitgescheiden worden door SPIα cellen. Helaas is het tot nu toe onmogelijk gebleken om de precieze identiteit van deze beschermende stoffen te achterhalen. Mocht u geïnteresseerd zijn in de zoektocht hiernaar, dan verwijs ik u graag naar het proefschrift van mijn collega, dr. Martijn Schenning. Wel is het zeer waarschijnlijk dat de bescherming werkt via het hierboven beschreven receptor/ligand systeem. De uitgescheiden beschermende stof (ligand) is waarschijnlijk een endocannabinoid (een gemodificeerde vetzuurstaart, zie kader) en de receptor waar deze stof op past de cannabinoid receptor.

Endocannabinoiden zijn lichaamseigen, lipid-achtige stoffen die, net als de werkzame stof in cannabis (THC, tetrahydrocannabinol), binden aan de cannabinoid receptor.
Naast bescherming, zorgt binding van een cannabinoid aan de cannabinoidreceptor (ofwel het activeren van het 'cannabinoidsysteem') ook voor een hongergevoel. Dit verklaart de neiging tot eten tijdens of na het blowen
De beschermende stof die onder invloed van PI-TPα gemaakt wordt, behoort waarschijnlijk tot groep endocannabinoiden die worden gemaakt van arachidonzuur. Dit is een vetzuurstaart die normaal gesproken onderdeel is van een lipide. Fosfatidylinositol, het lipide dat door PI-TPα door de cel vervoerd wordt, bevat veel arachidonzuur. Door het knip-eiwit (enzym) fosfolipase A_2 kan arachidonzuur van het lipide worden afgeknipt. Na een aantal omzettingsprocessen, onder andere uitgevoerd door het eiwit cyclooxygenase-2, is arachidonzuur omgezet tot een endocannabinoid. Bekende endocannabinoiden die van arachidonzuur zijn afgeleid, zijn anandamide en 2-arachidonoyl glycerol. Dit zijn echter niet de beschermende stoffen die onder invloed van PI-TPα gemaakt worden.

Hypothese

Omdat (i) PI-TPα veel in het CZS voorkomt, (ii) te weinig PI-TPα de oorzaak is voor neurodegeneratie en (iii) een hoge expressie van PI-TPα tot gevolg heeft dat

er beschermende stoffen worden gemaakt en uitgescheiden, was de hypothese van dit proefschrift dat hoge expressie van PI-TPα in het CZS essentieel is voor de overleving van (motorische) neuronen.

Resultaten

Om deze hypothese te onderbouwen, is in ***hoofdstuk 2*** onderzocht of de lipidachtige stoffen die door SPIα cellen worden uitgescheiden in staat zijn om motorische neuronen te beschermen tegen celdood. Dit bleek inderdaad het geval te zijn. Verder werd aangetoond dat de endocannabinoid receptor die in het celmembraan van de neuronen zit waarschijnlijk betrokken is bij de bescherming. Bovendien wordt in dit hoofstuk aangetoond dat PI-TPα in zowel motorische neuronen als in astrocyten tot expressie wordt gebracht.

Uit de resultaten van dit hoofstuk blijkt dus dat de PI-TPα-afhankelijke lipidachtige stoffen die door SPIα cellen worden uitgescheiden inderdaad in staat zijn om motorische neuronen te beschermen tegen celdood. Echter, SPIα cellen zijn in een laboratorium gemaakt en komen dus niet van nature in de hersenen voor. Dat betekent, mochten de PI-TPα-afhankelijke beschermende stoffen in het CSZ gemaakt worden, dat cellen in het CZS aanwezig moeten zijn die veel PI-TPα tot expressie brengen (genoeg om relevante hoeveelheden van de beschermende stoffen te produceren). Het was al bekend dat de PI-TPα expressie in het CZS hoog is, maar niet in welke soort hersencellen.

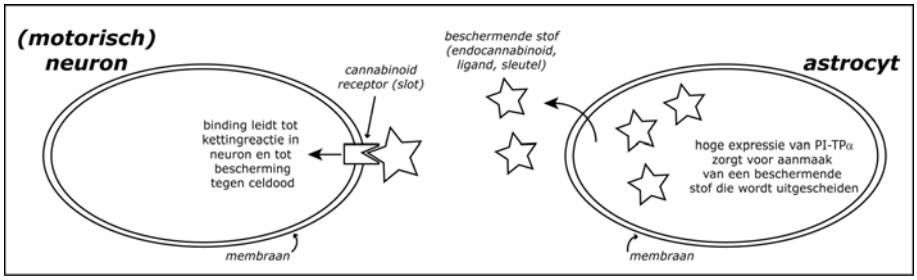
In ***hoofdstuk 3*** is het PI-TPα expressieniveau in verschillende soorten hersencellen onderzocht. Het niveau bleek hoog in astrocyten (minstens zo hoog als in SPIα cellen) en relatief laag in (motorische) neuronen. Dit wekte de suggestie dat de 'SPIα cellen van het CZS' waarschijnlijk de astrocyten zijn en niet de neuronen zelf.

Om dit te bewijzen, werden in ***hoofdstuk 4*** de stoffen die uitgescheiden worden door SPIα cellen, vergeleken met de stoffen die uitgescheiden worden door astrocyten. Het bleek dat beiden even goed in staat zijn om andere cellen (geen hersencellen) te beschermen tegen celdood. Het beschermingsmechanisme was ook vergelijkbaar (via de endocannabinoid receptor). Bovendien werd met behulp van massaspectrometrie (een techniek waarmee je de massa van verschillende stoffen die in een mengsel zitten, kunt meten) zichtbaar dat er drie PI-TPα-

afhankelijke stoffen uitgescheiden worden door zowel SPIα cellen als astrocyten. Helaas was het niet mogelijk de identiteit van deze stoffen te achterhalen. Echter, de eigenschappen van deze stoffen (retentietijd en massa/lading ratio) leken op die van verschillende bekende endocannabinoiden.
Om te onderzoeken of de beschermende stoffen die astrocyten uitscheiden daadwerkelijk afhankelijk zijn van hoge expressie van PI-TPα, werd in ***hoofdstuk 5*** een techniek toegepast die het expressieniveau van PI-TPα in astrocyten deed dalen naar ongeveer 10% van het normale niveau. Bij deze techniek (RNAi) worden stukjes RNA in de cel gebracht die specifiek plakken aan bijna al het RNA dat door de kern is uitgegeven om PI-TPα te maken. Hierdoor kan in het endoplasmatisch reticulum veel minder PI-TPα aangemaakt worden dan normaal.
Wanneer de hypothese zou kloppen, zouden deze cellen veel minder beschermenden stoffen moeten aanmaken. Dit werd getest op dezelfde cellen als in hoofdstuk 4 en op motorische neuronen. Het bleek dat de lipidachtige stoffen die uitgescheiden worden door astrocyten met minder PI-TPα inderdaad minder goed in staat zijn om beide celsoorten te beschermen tegen celdood.

Conclusie

De resultaten die beschreven zijn in dit proefschrift zijn samengevat in Figuur 7.1 en impliceren dat de productie en uitscheiding van PI-TPα-afhankelijke lipidachtige stoffen in het CZS inderdaad belangrijk lijken te zijn bij de overleving



Figuur 7.1: Schematische weergave van de resultaten zoals beschreven in dit proefschrift. In de astrocyt is de expressie van PI-TPα hoog, waardoor er beschermende stoffen (waarschijnlijk endocannabinoiden) aangemaakt worden. Deze worden uitgescheiden en binden aan de cannabinoid receptor die zich bevindt in het membraan van een (motorisch) neuron. De binding zorgt voor een kettingreactie in de cel die tot gevolg heeft dat het neuron beter beschermd is tegen celdood.

van neuronen.
Deze resultaten kunnen ook de degeneratie van neuronen in de vibrator en PI-TPα knock-out muis verklaren. Omdat in het CZS van deze muizen respectievelijk heel weinig of geen PI-TPα aanwezig is, worden ook geen PI-TPα-afhankelijke stoffen gemaakt. Hierdoor sterven eerst de motorische neuronen en neuronen in het cerebellum af. Deze twee soorten neuronen zijn namelijk de grootste en gevoeligste neuronen in het CZS. Omdat deze neuronen vitale organen (o.a. de longen) aansturen, gaan de muizen snel dood. Het feit dat de neuronen wel tot onwikkeling komen tijdens het embryonale stadium, kan worden verklaard met het feit dat de moedermuis wel een normale expressie van PI-TPα heeft en de beschermende stoffen via de gezamenlijke bloedsomloop bij de embryo's kan komen.
Ofschoon het mogelijk zou kunnen zijn dat andere glia cellen of zelfs de neuronen zelf (mede-) verantwoordelijk zouden kunnen zijn voor de productie en het uitscheiden van de PI-TPα-afhankelijke stoffen in het CZS, suggereren de hier beschreven experimenten dat astrocyten hier in ieder geval waarschijnlijke kandidaten voor zijn. Uiteraard is verder *in vivo* onderzoek (onderzoek in hele organismen) nodig om de hypothese van dit proefschrift te bewijzen.
Wanneer uit *in vivo* onderzoeken blijkt dat PI-TPα-afhankelijke stoffen inderdaad een rol spelen bij het beschermen van neuronen, en wanneer de identiteit van deze stoffen achterhaald wordt, dan zouden deze stoffen therapeutisch ingezet kunnen worden bij het bestrijden van neurodegeneratieve ziekten.
Verder is uit de experimenten die zijn beschreven in dit proefschrift gebleken dat de activatie van het cannabinoidsysteem belangrijk is voor de ontwikkeling en overleving van neuronen. Dit is een bevestiging van eerder gepubliceerde onderzoeken. Hieruit blijkt dat het *remmen* van de activatie van de cannabinoid receptor niet bevorderlijk zou zijn voor het welzijn van (motorische) neuronen. De auteur van dit proefschrift doet hierbij dan ook een oproep om terughoudend te zijn met het voorschrijven en gebruik van de dieetpil ***Acomplia™*** (rimonabant) die, door afsluiting van de cannabinoid receptor, zorgt voor een remming van het hongergevoel en daardoor gewichtsafname.

Dankwoord

Curriculum Vitae

List of publications

Dankwoord

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ooit nog eens zo'n 'pro' in wetenschappelijk schrijven te worden dat ik woorden als *to corroborate* en *concurrently* natuurlijk ga gebruiken...
Dop, het feit dat jij het mogelijk maakte dat ik aan het einde van mijn studie stage ging lopen in Sheffield heeft ervoor gezorgd dat ik terecht kwam in de wereld van de neurodegeneratieve ziekten. Bedankt daarvoor. Het eerste jaar bij neurologie heb ik als een mooi jaar ervaren waarin ik veel heb geleerd. Daarna hebben we elkaar door je nieuwe baan wat uit het oog verloren, om elkaar de laatste maanden weer tegen te komen. Bedankt voor het delen van je kennis.
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Tot de 15e!

Hanneke

Curriculum Vitae

De schrijfster van dit proefschrift werd op 9 september 1979 te Zeist geboren als oudste dochter van Albert en Els Bunte. Nadat er op O.B.S. De Koppel een solide educatieve basis was gelegd, werd deze verdiept op het Montessori Lyceum Herman Jordan in Zeist. In 1997 werd het V.W.O. diploma behaald en datzelfde jaar werd begonnen met de studie Scheikunde aan de Universiteit Utrecht. In 1998 werd het propedeusediploma behaald. In het derde studiejaar werd middels de onderzoeksoriëntatie de eerste 'laboratorium ervaring' opgedaan. Bij de sectie 'Biochemie van Lipiden' werd onder leiding van Prof. Op den Kamp en toen Drs. Rineke Steenbergen onderzoek gedaan naar veranderingen in pH en oxidantstatus van hartspiercellen tijdens ischemie en reperfusie. Gedurende het vierde jaar werd bij de Faculteit Geneeskunde, sectie 'Fysiologische Chemie', moleculair biologisch onderzoek verricht naar de transcriptiefactor pit-1 onder leiding van Prof. van der Vliet en toen Drs. Kevin Augustijn. Extracurriculair werd bij het IVLOS het vak 'Orientatie op het leraarschap' gevolgd. Vervolgens liep zij een 6 maanden durende stage bij de 'Academic Neurology Unit' van Sheffield University in Groot Brittannië waar onder leiding van Prof. Shaw en Dr. Allen een methode werd opgezet met het doel een eiwit biomarker te vinden in de cerebrospinale vloeistof van ALS patienten.
In 2002 werd het doctoraal diploma Scheikunde behaald en oktober van dat jaar werd als AIO gestart met het hier beschreven promotieonderzoek in dienst van de Universiteit Utrecht. Het project betrof een samenwerking tussen de secties 'Biochemie van Lipiden' (Instituut Biomembranen; Faculteit/ Departement Scheikunde) en 'Experimentele Neurologie' (Rudolf Magnus Instituut; Faculteit Geneeskunde).

List of publications

H. Bunte, M. Schenning, P. Sodaar, P.R. Bär, K.W.A. Wirtz, F.L. van Muiswinkel, and G.T. Snoek. A phosphatidylinositol transfer protein α-dependent survival factor protects cultured primary neurons against serum deprivation-induced cell death. *J. Neurochem.* 2006, 97(3), 707-715

H. Bunte, J. Vermeulen, P.R. Bär, K.W.A. Wirtz, G.T. Snoek, and F.L. van Muiswinkel. Phosphatidylinositol transfer protein α is involved in the secretion of a bio-active survival factor from C6 astroglioma cells.
Manuscript in preparation

H. Bunte, G.T. Snoek, P.R. Bär, K.W.A. Wirtz, and F.L. van Muiswinkel. Phosphatidylinositol transfer protein α and its role in neurodegeneration.
Manuscript in preparation