Universidade de Aveiro Departamento de Biologia
2007

MARIA DE FÁTIMA CAMÕES SOBRAL DE BASTOS

A NEUROTOXICIDADE DO ALUMÍNIO E SISTEMAS NEURONAIS DE FOSFORILAÇÃO

ALUMINIUM NEUROTOXICITY AND NEURONAL PHOSPHORYLATION SYSTEMS

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ALUMINIUM NEUROTOXICITY AND NEURONAL PHOSPHORYLATION SYSTEMS

tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Prof. Doutor Edgar Figueiredo da Cruz e Silva, Professor Associado do Departamento de Biologia da Universidade de Aveiro

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o júri

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palavras-chave

Alumínio, neurotoxicidade, expressão de proteínas, proteína fosfatase 1, neurofilamentos, cultura de células.

resumo

O alumínio é o terceiro elemento mais abundante na Terra. Uma vez que se encontra distribuído ubiquamente pelo meio ambiente e é utilizado em vários produtos e processos, a população humana está inevitavelmente exposta diariamente a este metal. De facto, o alumínio tem sido relacionado com diversas doenças neurodegenerativas como: a esclerose lateral amiotrófica, a demência de Parkinson (DP), a doença de Alzheimer (DA) e a encefalopatia relacionada com a diálise.

A fosforilação de proteínas é um dos principais mecanismos reguladores intracelulares da maior parte das vias de sinalização nas células eucarióticas. Este processo dinâmico regula o estado de fosforilação e/ou a actividade das proteínas através de um balanço entre as proteínas cinases, que fosforilam, e as proteínas fosfatases (PP) que desfosforilam as proteínas.

A proteína fosfatase 1 (PP1) é uma fosfatase específica para serina/treonina que está envolvida em importantes mecanismos celulares tais como o ciclo celular, contracção muscular e apoptose, entre outros. A PP1 tem três isoformas conhecidas, denominadas PP1α, PP1β e PP1γ,. O gene que codifica para a isoforma gama pode sofrer splicing alternativo originando a isoforma ubíqua PP1γ_1 e a isoforma enriquecida no testículo PP1γ_2.

A fosforilação anormal de proteínas tem sido associada a várias patologias, incluindo cancro, diabetes e várias doenças neurodegenerativas (DP, doença de Huntington e DA). Uma das proteínas que se encontram anormalmente fosforiladas na DA são, por exemplo, os neurofilamentos (NF).

Neste contexto, avaliou-se o efeito do alumínio na expressão de proteínas (PP1, NF) e realizou-se um estudo comparativo entre duas linhas celulares com características diferentes, as células PC12 e COS-1. Observou-se que o alumínio induziu um decréscimo na viabilidade celular, assim como na expressão e actividade de ambas as isoformas da PP1 em ambas as linhas celulares. Verificou-se que este efeito podia ser revertido retirando-se o alumínio. Um estudo semelhante foi ainda realizado num sistema neuronal utilizando culturas primárias de neurónios corticais. A expressão de ambas as isoformas da PP1 permaneceu inalterada após a exposição ao alumínio. No entanto, o alumínio induziu um decréscimo da expressão dos NF e da sinaptofisina, uma proteína que marca os terminais sinápticos. Por fim, estudou-se o efeito do alumínio na expressão de outras proteínas em sistemas *in vitro* e *in vivo* utilizando a tecnologia SELDI-TOF MS. Esta tecnologia permitiu detectar várias proteínas cuja expressão foi alterada devido ao alumínio. Com este trabalho pretendeu-se contribuir para um melhor conhecimento da neurotoxicidade do alumínio.

keywords

Aluminium, neurotoxicity, protein expression, protein phosphatase 1, neurofilaments, cell culture.

abstract

Aluminium is the third most abundant element on Earth. Aluminium is ubiquitous in the environment and is used in a variety of products and processes, thus, daily exposure of the general population to this metal is unavoidable. Indeed, aluminium has been implicated with various neurodegenerative disorders like: amyotrophic lateral sclerosis (ALS)/Parkinson's dementia (PD) complex of Guam, Alzheimer's disease (AD) and dialysis encephalopathy. Aluminium is known to interfere with several mechanisms of the nervous system, including alteration of cytoskeletal proteins, behavioural abnormalities, neurotransmission systems, oxidative damage, energy metabolism, second messengers, and also to induce neuronal apoptosis.

Protein phosphorylation is a major intracellular regulatory mechanism of all signalling pathways in the eukaryotic cell. This dynamic process regulates the net phosphorylation state and the activity of proteins by a balance between protein kinases, which phosphorylate, and protein phosphatases (PP), which dephosphorylate proteins. Protein phosphatase 1 (PP1) is a serine/threonine specific phosphatase which is involved in the control of important cellular mechanisms such as the cell cycle, muscle contraction and apoptosis, among others. PP1 has three known isoforms termed PP1α, PP1β and PP1γ. The gene for PP1γ produces by alternative splicing a ubiquitously expressed PP1γ1 and a testis-specific PP1γ2 isoform.

Abnormal protein phosphorylation has been associated with various disorders, including cancer, diabetes, and several neurodegenerative disorders (PD, Huntington's disease and AD). Besides tau other proteins that are abnormally phosphorylated in AD are the neurofilaments (NF).

In this context, the aluminium effect on the expression of proteins (PP1, NF) was evaluated. A comparative study was performed using two cell lines with different characteristics, PC12 and COS-1 cells. It was observed that aluminium induced a decrease in the cellular viability, as well as in the expression and activity of both PP1 isoforms in both cell lines. This effect was reverted following aluminium withdrawal. A similar study was also performed in a neuronal system, primary cortical neuron culture. The expression of both PP1 isoforms remained unchanged after aluminium exposure. However, aluminium induced a decrease in the expression of NF and of synaptophysin, a protein marker for synaptic terminals.

Finally, the effect of aluminium on the expression of other proteins in *in vitro* and *in vivo* systems was evaluated using SELDI-TOF MS technology. In this study, several proteins with altered expression due to aluminium were detected. This work aimed to contribute to the better understanding of aluminium neurotoxicity.

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PUBLICATIONS

Nesta dissertação foram utilizados resultados do trabalho publicado abaixo indicado. A autora declara que interveio na concepção e execução do trabalho experimental, na interpretação dos resultados e na sua redacção para publicação.

This thesis contains experimental results included in the publication indicated below. The author of this thesis declares that she participated in the planning and execution of the experimental work, as well as in data interpretation and in the preparation of work for publication.

Amador, Fátima Camões, Henriques, Ana Gabriela, da Cruz e Silva, Odete A. B., da Cruz e Silva, Edgar F., Monitoring protein phosphatase 1 isoform levels as a marker for cellular stress. *Neurotoxicol. and Teratol.* (2004) 26, 387-395.

ABBREVIATIONS

Abeta	Amyloid beta-peptide
ACN	Acetonitrile
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
Da	Dalton
DIV	Days in vitro
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
E18	Embryo with 18 days (pre-natal)
EAM	Energy absorbing molecule
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene glycol-*bis* (β-aminoethylether)-N,N,N',N'-tetra acetic acid
FBS	Fetal bovine serum
(x) *g*	Gravitational acceleration (when referring to centrifugation)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonate
HPLC	High pressure liquid chromatography
HRP	Horseradish peroxidase
HS	Horse serum
IC_{50}	50% Inhibition concentration
IgG	Immunoglobulin G

MAP	Microtubule-associated protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBT	Nitro blue tetrazolium
NF-nonP	Neurofilament nonphosphorylated
NF-H	Neurofilament heavy chain
NF-L	Neurofilament light chain
NF-M	Neurofilament medium chain
NF-P	Neurofilament phosphorylated
NFT	Neurofibrillary tangles
OD	Optical density
OGP	N-Octyl glucopyranoside
P4	Rat with 4 days (post-natal)
P7	Rat with 7 days (post-natal)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PD	Parkinson's disease
PHF	Paired helical filaments
PMSF	Phenyl methylsulfoxide
PP	Protein phosphatase
RT	Room temperature
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SPA	Sinapinic acid
Tau	Microtubule-associated protein τ
TBS	Tris buffered saline solution
TBS-T	Tris buffered saline-Tween 20
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl) aminomethane
WR	Working reagent

CHAPTER I

GENERAL INTRODUCTION

I. GENERAL INTRODUCTION

I.1. ALUMINIUM NEUROTOXICITY

I.1.1. Aluminium bioavailability, human intake and bioprocessing

Aluminium is a ubiquitous element used extensively in contemporary life. In spite of its abundance, aluminium is not an essential element and there is no known biological reaction that requires aluminium. However, the neurotoxicity of aluminium has been recognized for many years. Aluminium is highly abundant and the third most abundant element ubiquitously found in the natural environment, its bioavailability is high and is readily accessible. Humans are exposed daily to aluminium from different sources. Food is the main source of aluminium intake, while drinking water contributes only about 3% of total daily intake. Aluminium in drinking water has two main sources: dissolved aluminium is present naturally as a result of leaching from minerals in the soil and bedrock in the catchment of the water source, this leaching can be greatly enhanced as a result of acid precipitation; and, aluminium is widely used in water treatment as a coagulant, to reduce the number of small particles and to improve the colour of the water. It has been proposed that the safe amount of aluminium in drinking water should be lower than 0.1 mg/l (Martyn *et al*., 1989). Aluminium is found in beverages such as tea infusions which contain rather large amounts of aluminium, typically 2-6 mg/l (Flaten and Odegard, 1988), and is also found in processed food which contains aluminium-containing food additives. Food can also be contaminated with aluminium by contact through cooking devices, or when wrapped with aluminium foil, especially with the addition of acids such as lemon juice or vinegar. Aluminium is also present in canned drinks and canned food coming from the package (Jagannatha and Valeswara, 1995; Jagannatha, 1994). Humans are also in contact with aluminium through hygienic products like toothpaste and specially anti-perspirants, which have considerable amounts of aluminium that can be absorbed through the skin (Exley, 2004). In medicine, aluminium is used in several medications like

buffered aspirin, but especially in antacids used for the treatment of gastric ulcers, with typical consumption in the order of 1 g per day (Lione, 1985; Reinke *et al.*, 2003). Aluminium is also used in dental prothesis and as adjuvant in several vaccines (Brewer, 2006). Another potentially important source of exposure to aluminium is occupational exposure (McLachlan, 1995).

Exposure to aluminium is unavoidable during the entire life span. Humans consume an average of 7.6 mg/day of aluminium from drinking water and food (Yokel and McNamara, 2001). However, the absorption rate of aluminium is relative low, only 0.06-0.1% of ingested aluminium is absorbed across the gastrointestinal tract (Moore *et al.*, 2000). The gut is therefore the first barrier for aluminium uptake. Aluminium absorption is limited by the presence of certain other dietary components such as citrate, which can form a complex with the metal increasing significantly its absorption (Whitehead *et al.*, 1997). Age contributes to gut barrier impairment and so to the aluminium absorption. Indeed, it was reported that young individuals absorb much less aluminium from an aluminium citrate drink than older people (Taylor *et al.*, 1992). In the bloodstream, aluminium binds to the plasma proteins, transferrin (the main protein carrier) and albumin, the remainder complexes with citrate (the main small molecule carrier) (Martin *et al.*, 1987; Fatemi *et al.*, 1991). Aluminium in circulation may distribute through all the body. The main target for accumulation is the skeleton (Kerr *et al.*, 1992), although, liver, kidney, muscle and heart also accumulate aluminium (Walker *et al.*, 1994). It has been proposed that aluminium could enter the brain from systemic circulation by three different routes: blood-brain barrier (BBB), nasal-olfactory pathway (by olfactory nerves) and cerebrospinal fluid (through the choroid plexus) (Perl and Good, 1987; Yokel *et al.*, 1999). The BBB route is generally considered to be the most plausible mode of entry (Yokel, 2002). The potential mechanisms of distribution of substances across the BBB, the second barrier to aluminium, are the same as those across any cell membrane: diffusion, carrier/receptor mediated transport by facilitated diffusion and active transport. One of the most probable mechanisms of aluminium access into the brain is by transferrin-receptor-mediated endocytosis (Roskams and Connor, 1990), the iron transport protein by excellence. Another important carrier for brain aluminium influx may be monocarboxylate transporter, a proton co-transporter located at the BBB (Gerhart *et al.*, 1997; Yokel *et al.*, 2002). However, aluminium alone affects the permeability of the BBB enhancing its

apparent lipophilicity, thus contributing to an increase of its transmembrane diffusion (Meiri *et al.*, 1993), although this depends on the physicochemical properties of the metal coordination sphere (Favarato *et al.*, 1992). In the brain, aluminium competes with other elements, such as Fe, Ca and Mg, present in several proteins and enzymes altering their function. Indeed, some aluminium persists in the brain for a long time. Brain aluminium efflux, presumably Al-citrate, was suggested to be mediated by the monocarboxylate transporter, through the BBB (Yokel *et al.*, 1999). Absorbed aluminium is primarily eliminated via the kidneys and approximately 2% excreted in bile (Alfrey, 1986a; Yokel and McNamara, 2001).

I.1.2. Aluminium speciation chemistry

The chemical speciation of aluminium in aqueous solution is of particular interest, as the form of aluminium regulates its solubility, bioavailability and consequently its toxicity. One factor determining the form of aluminium in solution is pH (Figure I.1). Aluminium is a strong hydrolyzing element and is generally insoluble at neutral pH. Its solubility is enhanced under acidic or alkaline conditions and in the presence of appropriate ligands. Thus, in the range of physiologic pH values (between 6 and 8) aluminium is generally insoluble.

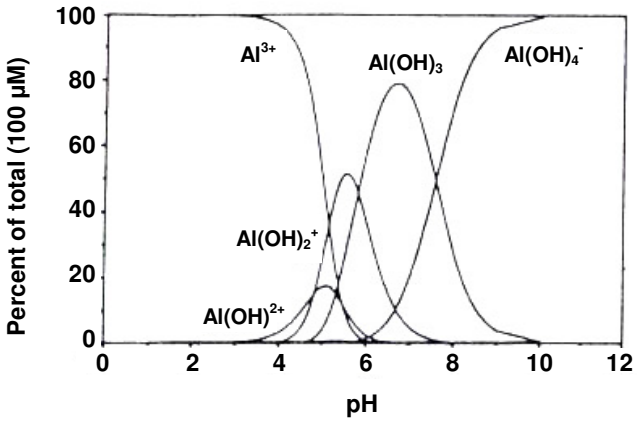

Figure I.1 – Distribution of the hydrolysis products of aluminium as a function of pH (adapted from Meiri *et al.*, 1993).

In acidic solutions (pH<5), aluminium exists as an octahedral hexahydrate, $Al(H_2O)_6^{3+}$, usually abbreviated as Al^{3+} and sometimes referred in the literature as free aluminium. As pH increases, $Al(H_2O)_6^{3+}$ undergoes successive deprotonations to yield different species such as $Al(OH)^{2+}$ and $Al(OH)_2^{+}$ with decreasing solubility. In neutral pH solutions, the amorphous $Al(OH)_3$ is produced and precipitates. At alkaline pH this precipitate redissolves to form tetrahedral aluminate, $Al(OH)_4^-$, the primary soluble aluminium species at a biological pH>6.2. The main species at pH<5 is the octahedral hexahydrate, $Al(H_2O)_6^{3+}$ and at pH>6.2 the tetrahedral $Al(OH)_4^-$. At pH between 5 and 6.2 all species co-exist. Thus, at pH 7.4 the main species is the insoluble $Al(OH)_3$ although, the prominent soluble species is the $Al(OH)_4^-$ (Martin, 1986; Meiri *et al.*, 1993; Gupta, 2005).

It should be pointed out that a solution of $AlCl_3$ at physiological pH contains free aluminium (Al^{3+}) at concentrations much lower than the indicating concentration, because $AlCl_3$ forms insoluble hydroxyl complexes at that pH. Indeed, when aluminium inorganic salts, such as chloride, sulphate, hydroxide or perchloride, are dissolved in water at a calculated concentration of 10 mM, the aluminium concentration is about 50 μM. The use of Al-lactate or Al-aspartate increases the soluble aluminium concentration to approximately 55-330 μM and the use of Al-maltolate or gluconate increases the soluble aluminium concentration to 4-6 mM. Aluminium complexes of low solubility can be biologically relevant; however, free aluminium is the species which is bioactive, the species that links to proteins or ligands, even at low concentration (Martin, 1986; Meiri *et al.*, 1993; Gupta, 2005). Thus, the solution concentration is merely informative, as at physiological pH the bioactive aluminium concentration is much lower.

Aluminium bioavailability, concerning diet and intestinal absorption, depends on which complexes it forms. Several compounds in the diet, including ascorbic acid, citric acid, lactic acid and malic acid, may increase aluminium absorption in the intestine by elevating the pH of aluminium hydroxide precipitation (Partridge *et al.*, 1989). On the other hand, phosphate is also an important dietary factor, forming complexes even at low pH and making aluminium less available for absorption (Driscoll and Schecher, 1988). It has been suggested that the presence of phosphates in the diet is probably the "natural" mechanism whereby aluminium is prevented from entering the circulation (Martin, 1986). Aluminium has also been reported to displace other ions of physiological relevance.

Aluminium is a small ion with an ionic radius of 54 pm and can replace divalent metals such as Ca, Mg and Zn, whose ionic radii are 72, 74 and 100 pm, respectively, and hence is thought to be responsible for executing various toxic effects (Martin, 1996).

Aluminium binds strongly to oxygen-donor ligands, particularly if they are negatively charged. Inorganic or organic phosphates, carboxylate and deprotonated hydroxyl groups are strong Al^{3+} (aluminium) binders. Thus, aluminium binds to the phosphor groups of DNA or RNA, influences DNA topology and affects gene transcription (Lukiw *et al.*, 1998). Phosphate groups of cell membranes are also targets for aluminium binding (Van Rensburg *et al.*, 1995). As well as phosphorylated proteins, aluminium has been reported to influence various functions of enzymes including protein kinases and phosphatases (Shetty *et al.*, 1992; Amador *et al.*, 2004). Overall, the form and speciation of aluminium may be critical to its biological actions.

I.1.3. Aluminium and neurodegenerative diseases

Acute aluminium exposure is of low toxicity. In humans, oral doses up to 7.2 mg/day are routinely tolerated without any signs of harmful short-term effects. However, intake of large amounts of aluminium can lead to a wide range of toxic effects, including microcytic anaemia (Touam *et al.*, 1983; Jeffery *et al.*, 1996; Garbossa *et al.*, 1998), osteomalacia (Bushinsky *et al.*, 1995; Jablonski *et al.*, 1996; Jeffery *et al.*, 1996), glucose intolerance of uraemia (Banks *et al.*, 1987) and cardiac arrest (Starkey, 1987). Elderly persons with elevated serum aluminium levels exhibit impaired complex visual-motor co-ordination and poor long-term memory (Bowdler *et al.*, 1979).

Evidence suggests that trace metal homeostasis plays a crucial role in the normal functioning of the brain and any disturbance in it can exacerbate events associated with neurodegenerative disorders. In fact, aluminium has been implicated in several neurological and other disorders, namely dialysis encephalopathy (Alfrey *et al.*, 1976; Savory and Wills, 1984; Kerr and Ward, 1988), amyotrophic lateral sclerosis (ALS)/Parkinsonism-dementia (PD) complex of Guam (Perl *et al.*, 1982; Kurland, 1988) or Alzheimer's disease (AD) (Perl and Brody, 1980; Perl, 1988; Xu *et al.*, 1992a).

I.1.3.1. Dialysis encephalopathy

Dialysis encephalopathy is a fatal brain disorder occurring in some patients with chronic renal failure undergoing inadvertent parenteral exposure to aluminium (Alfrey *et al.*, 1980). The chronic symptoms include speech disorders, neuropsychiatric abnormalities and multifocal myoclonus (Dewberry *et al.*, 1980). More subtle symptoms of the condition include disturbances of tetra-hydrobiopterin metabolism and abnormalities in a number of psycho-motor functions (e.g., visual spatial recognition memory), all occurring at mildly elevated serum aluminium levels (59 µg/l) and in the absence of chronic dementia (Altmann *et al.*, 1989). Patients with dialysis dementia were shown to have markedly elevated serum aluminium levels with increased concentrations in many tissues namely, kidney, liver, bone, heart and throughout the cerebral cortex (Alfrey *et al.*, 1980; Kerr and Ward, 1988; Meiri *et al.*, 1993). Investigators reported a correlation between the aluminium concentration in water used to prepare the dialysate fluid and the incidence of dialysis dementia (Savory and Wills, 1984). Tissue accumulation of aluminium to levels high enough to cause toxicity is mainly due to a combination of high exposure, partly directly into bloodstream (thus bypassing absorption in the gastrointestinal tract) and these patients' lack of kidney function, which is the main excretion route for aluminium. The mechanism of neurotoxicity in dialysis encephalopathy has not been established. However, severe acute aluminium intoxication cases have been reported to respond to chelation therapy with desferrioxamine to lower serum aluminium, combined with hemodialysis (Vaan Landeghem *et al.*, 1997; Nakamura *et al.*, 2000). Indeed, desferrioxamine has been reported to accelerate clearance of brain aluminium (Yokel *et al.*, 2001). It was also proposed that ascorbate, desferrioxamine and Ferralex-G in combination as a "molecular shuttle chelation" may provide a useful pharmacotherapy in the potential treatment of aluminium overload disease (Kruck *et al.*, 2004). Dialysis encephalopathy syndrome resulting from acute intoxication of aluminium caused by the use of an aluminium-containing dialysate was common occurrence prior to 1980. However, using modern techniques of water purification, such acute intoxication can now be avoided (Rob *et al.*, 2001). Nevertheless, aluminium toxicity is a known adverse effect in patients with end-stage renal disease due to oral intake of aluminium-containing phosphate binders (Wills and Savory, 1989). Indeed, a fatal case of aluminium

encephalopathy in a patient with severe chronic renal failure not on dialysis but due to intake of large doses of antacids containing aluminium for at least 3 years, was recently reported (Zatta *et al.*, 2004). A similar report of an autopsy of a 59-year-old female aluminium encephalopathy patient who had chronic renal failure and took 3.0 g hydroxy-aluminum gel per day for the control of serum phosphorus level during a 15-year period, was published by Shirabe and colleagues (2002). Furthermore, it was reported recently significant aluminium toxicity in a non-hemodialysis patient who chronically injected intravenously oral methadone solution heated in an aluminium-based cooking utensil (Friesen *et al.*, 2006).

The aluminium contamination of total parenteral nutrition (TPN) solutions is also a matter of great concern. It was reported that the neurological development of premature infants who had received a TPN solution containing a high level of aluminium was impaired compared with infants who had received an aluminium-depleted TPN solution (Bishop *et al.*, 1997). Considering that aluminium in TPN solutions is highly bioavailable and that renal function of infants is impaired, the aluminium contamination of TPN solutions may cause serious brain damage (Kawahara, 2005).

I.1.3.2. Amyotrophic lateral sclerosis and Parkinsonism-dementia complex of Guam

It has been postulated that aluminium plays a role in the aetiology of two severe neurodegenerative diseases, amyotrophic lateral sclerosis (ALS) and Parkinsonism-dementia (PD) complex of Guam. ALS/PD are characterized by the selective degeneration of motor neurons, the presence of neurofibrillary tangles (NFT) and neuropil threads in the brain, these features result in a clinical syndrome of progressive weakness, culminating in respiratory failure and death (Wakayama *et al.*, 1993a). Both diseases are observed at very high incidence among the Chamorro people of Guam (Garruto *et al.*, 1990; Oyanagi, 2005). A high incidence of ALS is also found in two other areas, western New Guinea and the Kii Peninsula of Japan (Yase *et al.*, 2001). The soils and drinking water of Guam and the two other affected areas are very low in calcium and magnesium but very high in aluminium, iron and silicon (Gajdusek and Salazar, 1982). Aluminium was found to accumulate in the tangle-bearing neurons in post-mortem brains of patients with ALS/PD

(Perl *et al.*, 1982). Garruto and Yase (1986) suggested that chronic nutritional deficiencies of calcium and magnesium may lead to increased absorption of aluminium (and other metals), resulting in the deposition of aluminium in neurons. These deposits could interfere with the structure of neurons and eventually result in NFT (Garruto, 1989). The elevated concentrations of aluminium in ALS/PD patients suggested that aluminium and the depletion of calcium and magnesium may play key roles in the pathogenesis of ALS/PD (Yase *et al.*, 2001). The dramatic decrease in the incidence of ALS/PD on Guam with a change in dietary habits and local water supplies has given support to this theory (Garruto *et al.*, 1990). Although, the remarkable clustering of motor neuron diseases (MND) was thought to have disappeared, the southern Kii Peninsula remains a high-risk area for MND, especially if the emigrants who developed MND one to four decades after leaving the focus are included (Yoshida *et al.*, 1998). It has been reported that experimental animals chronically fed a low-calcium and/or magnesium and high-aluminium diet showed neuronal loss in the spinal anterior horn and cerebral cortices (Florence *et al.*, 1994). Further mice with a similar diet (low-Ca/Mg and high-Al) exhibited the deposition of aluminium, the deposition of hyperphosphorylated tau proteins (the basic structure of NFT) and neuronal loss (Kihira *et al.*, 2002). Recently, it was also reported that mice fed a similar diet also exhibited ALS-like skin and CNS changes (Kihira *et al.*, 2004).

I.1.3.3. Alzheimer's disease

The association of aluminium with Alzheimer's disease (AD) has more than 25 years, however it is still a controversial issue and the mechanisms of aluminium toxicity in this disease are not yet established. AD is a neurological disorder affecting elderly people, as first described by Alois Alzheimer in 1906. Patients with AD exhibit progressive mental deterioration manifested by memory loss, inability to calculate, visual-spatial disturbances, confusion and disorientation. The neuropathological characteristics include: cortical and subcortical atrophy; intraneuronal accumulation of neurofibrillary tangles (NFT), which are composed of paired helical filaments (PHF) of hyperphosphorylated tau proteins (Figure I.2); dystrophic neurites surrounding the extracellular deposits of amyloid beta-peptide (Abeta) in plaques (neuritic plaques or senile plaques) (Figure I.2); formation

of neuropil threads; loss of synaptic function; oxidative stress and apoptosis, leading to neuronal loss (Glenner and Wong, 1984; Grundke-Iqbal *et al.*, 1986a; Markesbery, 1997; Christen, 2000; Dickson, 2004; LeBlanc, 2005). These events are observed mostly in the hippocampal and cortical regions of AD brains. The etiological factors of AD are not clearly elucidated, although current hypotheses include genetics, head trauma, oxidative stress, infectious agents and environmental factors, including aluminium toxicity.

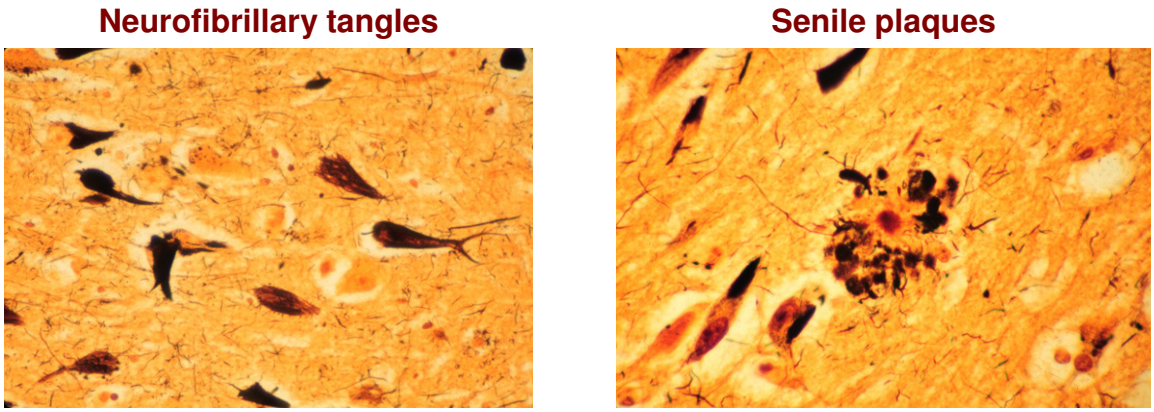

Figure I.2 – Neurofibrillary tangles and senile plaques. In both AD hallmarks aluminium was found to accumulate.

One of the first studies relating aluminium with AD was published in 1973 by Crapper and colleagues (Crapper *et al.*, 1973). In this study they demonstrated that AD brain tissues showed a 2 to 3-fold increase in aluminium concentrations when compared to normal control tissues. Later, it was reported that the aluminium content of human brain is around 6.2-9.8 μg/g (dry mass brain) (Xu *et al.*, 1992a). However, it has been reported that AD patients have elevated concentrations of aluminium (9.0-11.0 μg/g dry weight) in some regions (cortex, mesial temporal and temporal cortex) of their brains compared to controls (Crapper *et al.*, 1973; Solomon *et al.*, 2001; Andrasi *et al.*, 2005; Gupta *et al.*, 2005). Aluminium tends to accumulate more in the cortex and hippocampus, both in normal and AD brains (McDermott *et al.*, 1979). Aluminium deposition is progressive, being higher in severe AD than in moderate AD and relatively low in normal brain (Jagannatha *et al.*, 1999). Even though, the increased content of aluminium in NFT has been established (Perl and Brody, 1980; Lovell *et al.*, 1993; Shin *et al.*, 1994; McLachlan, 1995; Tokutake *et al.*, 1995), the presence of aluminium and silicon in the central region of

senile plaque cores in the cortex of AD patients is a controversial issue. Landsberg *et al.*, (1992) stated that they could not find aluminium in senile plaques from autopsy AD brain material, and hypothesized that the occurrence of aluminium found previously in plaques by Candy and colleagues (1986) had been caused by contamination from dyes used to stain the plaques. However, Good and Perl (1993) stated that the Landsberg and co-workers (1992) study did not contradict the aluminium hypothesis, since it was clear from the literature that aluminium was more often associated with the NFT than with the plaques (Zatta, 1993). Indeed, Tokutake and co-workers (1995) found aluminium contained in lipofuscin granules with silicon, probably as aluminosilicate, in senile plaques of brain with AD. Moreover, Exley (2005) reported the detection of aluminium associated with Abeta in AD brains. Overall, whether the presence of aluminium in the brain of AD patients is a cause or a result of the condition remains unknown.

Moore and co-workers (2000) showed that under normal physiological conditions, the ability of the gastrointestinal tract to exclude aluminium is reduced in AD, possibly leading to greater systemic exposure to aluminium. Additionally, a higher frequency of a genetic variant of transferrin, TfC2, has been found in AD patients compared with non-demented controls, suggesting that this factor may be involved in an aberrant transport of aluminium in these patients (Van Rensburg *et al.*, 1995; Zambenedetti *et al.*, 2003). Moreover, ferritin, the iron storage protein, isolated from the brains of AD subjects, has 6-fold higher aluminium content than normal age-matched controls (Fleming and Joshi, 1987). Indeed, aluminium has been shown to accumulate in rat brain ferritin (Sakamoto *et al.*, 2004), which has been reported to be a component of the senile plaques in AD (Grundke-Iqbal *et al.*, 1990). From this evidence we may conclude that the aluminium absorption, uptake and accumulation are augmented in patients with AD. On the other hand, the incidence of AD is increased in regions where people are more exposed to aluminium. Although, drinking water contributes only with a minor portion of the total daily oral intake of aluminium, it was found that a significant relationship exists between the number of AD cases and the levels of aluminium present in drinking water (McLachlan *et al.*, 1996; Gauthier *et al.*, 2000; Flaten, 2001). Additionally, two metal chelation therapies have been proposed for AD patients, one using Feralex-G, and another using desferrioxamine (Shin *et al.*, 2003; House *et al.*, 2004) A "molecular shuttle chelation" using both desferrioxamine and Feralex-G in combination with ascorbate was also

proposed as a therapy for aluminium overload diseases that may include AD patients (Kruck *et al.*, 2004).

The aluminium hypothesis has also been disputed based on the following features: (i) not all patients with AD have high brain levels of aluminium and the senile plaques that are common in AD are not seen in experimental aluminium toxicity (Wisniewski *et al.*, 1980; Bjertness *et al.*, 1996); (ii) the incidence of cognitive impairment and AD symptoms is not increased, but only dialysis dementia in renal patients is observed with increased aluminium levels (Alfrey, 1986b); (iii) it has been shown that NFT in AD, which are composed primarily of paired helical filaments (PHF) are made up mainly of hyperphosphorylated tau (a microtubule associated protein), in contrast to aluminium-induced neurofibrillary degeneration (NFD), which consists of aggregated hyperphosphorylated neurofilament (an intermediate filament of the neuronal cytoskeleton) (Munoz-Garcia *et al.*, 1986; Erasmus *et al.*, 1993). However, abnormally phosphorylated tau has been found in aluminium-induced neurofilamentous aggregates, together with abnormally phosphorylated neurofilament protein (Singer *et al.*, 1997; Huang *et al.*, 1997). Also, elevated levels of phosphorylated neurofilament proteins were found in cerebrospinal fluid of AD patients (Hu *et al.*, 2002). In addition, immunoreactivity to phosphorylated epitopes of neurofilaments was observed in AD tangles (Sternberger *et al.*, 1985). Nevertheless, the neurotoxic effects of aluminium are beyond any doubt, and aluminium as a factor in AD cannot be discarded, especially concerning the most elderly (over 75), or until the uncertainty about the neuropathological evidence is resolved (Gupta *et al.*, 2005). Consequently, as a result of continued concern about the neurotoxicity of aluminium, the U.S. Environmental Protection Agency has put aluminium on its contaminant candidate list (U.S. Environm. Prot. Agency, 2002), the U.S. Food and Drug Administration implemented labelling requirements for aluminium in large and small volume parenterals (US Food and Drug Adm., 2002) and Canada established operational guidance limits for drinking water aluminium on the basis of the precautionary principle (Health Canada, 2002).

I.1.4. Effects of aluminium on nervous system

The mechanisms by which aluminium induces neurotoxicity still remain to be elucidated. However, a wide range of aluminium effects have been reported in relation to the neuronal response to aluminium exposure. Intraneuronal neurofilamentous aggregates formed due to aluminium have been observed, mainly in rabbits. In rodents a number of neurochemical and neurophysiological alterations following *in vivo* or *in vitro* exposure to aluminium have been observed. Aluminium has been shown to affect behaviour, cholinergic activity, lipid peroxidation, glucose metabolism and signal transduction.

I.1.4.1. Cytoskeletal protein aggregates

Intracisternal inoculation of aluminium into rabbit brain induces intraneuronal neurofilamentous aggregates (Klatzo *et al.*, 1965; Savory *et al.*, 1999; He and Strong, 2000). Injected rabbits had neurological symptoms with paralysis of their skeletal muscles and died with tetanic spasm just over 10 days after the administration of aluminium (Gotow, 2000). Aluminium-induced neurofilamentous aggregates are characterized by argentophilic masses in neuronal perikarya (area surrounding the nucleus), proximal axonal enlargements and proximal dendrites, which are aggregates of abnormally phosphorylated neurofilament proteins (Troncoso *et al.*, 1986; Gotow *et al.*, 1995; Gotow, 2000). It was suggested that there is a relationship between the phosphorylation state and the structural organization of those neurofilaments (Gotow *et al.*, 1995). Abnormally phosphorylated tau has also been found in aluminium-induced neurofilamentous aggregates in rabbits (Savory *et al.*, 1996; Singer *et al.*, 1997; Huang *et al.*, 1997). Savory and co-workers (1996) studied the time course of cytoskeleton protein phosphorylation in aluminium injected animals and found that the argyrophilic bodies appeared 24 h after aluminium maltolate administration, with a predominance of neurofilament proteins. Non-phosphorylated, phosphorylation independent epitopes appeared first, followed at about 72 h by phosphorylated forms. Tau was also detected at the 72 h mark, although the characteristic epitopes of AD become most distinct at 6-7 days following aluminium injection (Savory *et al.*, 1996). In addition, aluminium-intoxicated rabbits also exhibit dendritic degeneration in motor neurons (Wakayama *et al.*, 1993b).

The direct injection of aluminium compounds into the rabbit central nervous system mimics abnormalities found in human neurodegenerative diseases. The rabbit/aluminium model system provides a means of elucidating mechanisms of neurodegeneration (Bharathi *et al.*, 2006), particularly those involving apoptosis and abnormal cytoskeletal proteins (Savory *et al.*, 1999, 2003; He and Strong, 2000; Ghribi *et al.*, 2001a). Rabbits inoculated intracisternally with aluminium exhibited many of the clinical, histological and ultrastructural characteristics of ALS including argentophilic perikaryal inclusions and neurofibrillary tangle-like morphologies (Wakayama *et al.*, 1996). The altered conditions observed in aluminium injected rabbits also mimic a number of neuropathological and biochemical changes present in AD and related human neurodegenerative disorders, like amyloid precursor protein, Abeta, alpha 1-antichymotrypsin and ubiquitin-like immunoreactivities in neurofibrillary degeneration-bearing neurons (Huang *et al.*, 1997). It has been proposed that phosphorylation of cytoskeletal proteins induces the formation of neurofilamentous aggregates, particularly in human neurodegenerative disorders. Given that these aggregates are hyperphosphorylated, phosphorylation alone would make these protein accumulations unstable because of the predominance of negative charges on the phosphate groups. Therefore it can be postulated that a positively-charged species would represent an inherent factor for both the formation and stabilization of the neurofibrillary aggregates, in AD as well as in experimental aluminium-induced neurofibrillary degeneration; aluminium is a strong candidate for this role in the latter (Savory *et al.*, 2001).

I.1.4.2. Behavioural abnormalities

Animals exposed to aluminium exhibit behavioural abnormalities like spatial disorientation, lower activity and higher emotionality (Miu *et al.*, 2003; Roig *et al.*, 2006). Deficits in cognitive and motor function (Oteiza *et al.*, 1993), as well as changes in learning and memory (Julka *et al.*, 1995; Kaneko *et al.*, 2006), have also been noted. In a study using young and old rats exposed to aluminium, while no significant effects of aluminium exposure between groups could be detected on behaviour, the total number of synapses in the CA1 fields of hippocampal decreased with age and aluminium exposure (Colomina *et al.*, 2002). Platt and co-workers (2001), using histochemical and

immunocytochemical studies, suggest that the enhancement of inflammation and the interference with cholinergic projections may be the mode of action through which aluminium causes learning and memory deficits. Additionally, an aluminium impairment of hippocampal long-term potentiation, a model for synaptic plasticity underlying some forms of learning and memory, has been reported in rats both *in vivo* and *in vitro* (Platt *et al.*, 1995).

I.1.4.3. Cholinergic and other neurotransmission systems

The cholinergic system is an important component of the neuronal circuitry of learning and memory mechanisms (Alkon *et al.*, 1991; Cain, 1998). Aluminium alters the cholinergic transmission, which is reflected in neurobehavioral deficits (Julka *et al.*, 1995). Significant decrease in choline acetyltransferase (ChAT) activity after chronic aluminium treatment has been observed in the parietal cortex, hippocampus and striatum of rat brain (Gulya *et al.*, 1990). Following aluminium exposure the inhibition of ChAT, the reduction of acetylcholine levels and a significant decrease in high-affinity choline uptake were observed (Julka *et al.*, 1995). It was also reported that under oxidizing conditions aluminium potentiated the inhibition of the high-affinity choline uptake observed following lipid peroxidation (induced by ascorbate/iron) (Amador *et al.*, 2001). The effect of aluminium on the acetylcholinesterase (AChE) activity is a controversial issue. An activation of AChE by aluminium *in vivo* and to a lesser extent *in vitro* was reported by Zatta *et al.* (2002). However, an inactivation of the same enzyme was reported by Julka and co-workers (1995). It was suggested that the difference in activity indicates that aluminium speciation may play a relevant role in producing toxicological effects (Zatta *et al.*, 2003). Indeed, prolonged treatment with aluminium chloride resulted in inhibition of AChE in rat brain (Dave *et al.*, 2002). However, Kaizer and co-workers (2005) verified an activation of AChE in different mouse brain regions after exposure to aluminium and citrate. Still, the AChE activity in mice exposed only to aluminium was verified inhibited in the hypothalamus and enhanced in the striatum.

Various authors reported the interference of aluminium with other neurotransmission systems namely: glutamatergic (Platt *et al.*, 1994; Nayak and Chatterjee, 2001; Yang *et al.*, 2003), GABAergic (Trombley, 1998; El-Rahman, 2003), serotonergic

(Kumar, 2002) and dopaminergic systems (Tsunoda and Sharma, 1999; Milanese *et al.*, 2001).

I.1.4.4. Oxidative damage

Aluminium, a metal without redox capacity in biological systems, has been shown to exacerbate oxidative damage initiated by reactive oxygen species (ROS) generating systems. Aluminium has been shown to promote iron-induced lipid peroxidation (Gutteridge *et al.*, 1985) and to potentiate lipid peroxidation induced by ascorbate/iron inducing system (Amador *et al.*, 2001). Aluminium promotion of melanin-initiated oxidative damage was also demonstrated (Meglio and Oteiza, 1999), as well as the lipid peroxidation initiated by xanthine/xanthine oxidase system, another ROS generating system (Golub *et al.*, 2002). Mundy and co-workers (1997) reported that aluminium pre-treatment potentiates the ROS production induced by iron in primary neuronal cultures. Moreover, the concentration of lipid peroxidation products was found to increase in rat brain following aluminium lactate injections (Ogasawara *et al.*, 2003). Rats treated similarly exhibited changes on oxidative stress markers (glutathione transferase, glutathione reductase and peroxidase, reduced and oxidized glutathione, superoxide dismutase, catalase and thiobarbituric acid reactive substances) in different neural areas, indicating that aluminium acts as pro-oxidant (Esparza *et al.*, 2003). Orally administered aluminium-maltolate complex was shown to enhance oxidative stress in the organs of mice (Kaneko *et al.*, 2004). More recently, chronic aluminium exposure in drinking water was observed to specifically enhance oxidative, as well as inflammatory events in the mouse brain (Becaria *et al.*, 2006). Enhanced lipid peroxidation after long-term exposure to low levels of aluminium on different mouse brain regions was also reported (Kaizer *et al.*, 2005). Increased aluminium in plasma and erythrocytes, and increased superoxide dismutase activity in erythrocytes of rat exposed to aluminium was also described (Guo *et al.*, 2004). The mechanisms proposed for aluminium promotion of lipid peroxidation involve alterations in lipid substrates that enhance their susceptibility to oxidative damage, and changes in the physical properties of membranes (Oteiza, 1994; Van Rensburg *et al.*, 1995). The mechanism is based on aluminium binding to phospholipid headgroups within the cell membrane and promotion of changes in the arrangement of membrane lipids,

including packing of fatty acids which facilitate the propagation of lipid peroxidation (Oteiza, 1994). The aluminium-induced changes in the membrane physical properties include alterations of membrane fluidity and lipid rearrangement through lateral phase separation (Verstraeten *et al.*, 2002). Indeed, lipid peroxidation was shown to facilitate aluminium accumulation in rat nerve terminals (Amador *et al.*, 1999). The alteration of the structure and function of cell membranes induced by aluminium was also reported in human erythrocytes, leading to alterations of its shape (Suwalsky *et al.*, 2004).

I.1.4.5. Energy metabolism

Due to its high reactivity, aluminium is able to interfere with several biological functions, including enzymatic activities in key metabolic pathways. Thus, aluminium may compromise energy production via the Krebs cycle by activating α-ketoglutarate dehydrogenase and succinate dehydrogenase, while inhibiting aconitase (Zatta *et al.*, 2000). Glucose metabolism is also impaired by aluminium which is a strong inhibitor of some enzymes of the glycolysis pathway. Aluminium inhibits the activities of hexokinase and glucose-6-phosphate dehydrogenase (Cho and Joshi, 1989; Exley *et al.*, 1994). Moreover, a reduction in glucose metabolism in rat brain following chronic aluminium exposure was observed (Clauberg *et al.*, 1994). However, recently Kaur and Gill (2006) reported that chronic aluminium exposure enhanced the activity of glucose-6-phosphate dehydrogenase.

I.1.4.6. Signal transduction pathways (second messengers)

Signal transduction pathways, including inositol 1, 4, 5-triphosphate (IP_3) and cAMP-mediated signalling, appear to be targets of aluminium action both *in vivo* and *in vitro*. These signalling pathways regulate important functions such as cell differentiation and proliferation, neurotransmitter release and synaptic plasticity. Moreover, IP_3 is also involved in long-term potentiation, i.e. a mechanism underlying memory formation (Berridge, 1986). The IP_3 signalling system starts with the binding of a neurotransmitter or other ligand to a receptor with the consequent activation of the enzyme

phosphatidylinositol-4, 5-diphosphate (PIP_2)-specific phospholipase C (PLC), mediated by a guanine nucleotide-binding protein (G-protein). Activated PIP_2-PLC catalyses the hydrolysis of PIP_2 into the second messengers diacylglycerol and IP_3. Aluminium was found to inhibit receptor-stimulated IP_3 production in neuroblastoma cells, in a concentration-dependent manner (Shi and Haug, 1992; Shi *et al.*, 1993). Although, it was suggested that receptor, G-protein, or receptor-G-protein interactions are not affected by aluminium (Shafer *et al.*, 1993), the competitive inhibition of PIP_2-PLC by aluminium (100 μM $AlCl_3$ or aluminium lactate) was verified in different rat brain regions (Nostrandt *et al.*, 1996). Indeed, aluminium at 500 μM was reported to inhibit the PIP2-PLC activity by approximately 80% (Shafer *et al.*, 1994). The aluminium inhibition of PLC and consequent decrease on IP_3 accumulation is not age-dependent, as verified by the similar response obtained from cortical homogenates of 7 day old and adult rats (Mundy *et al.*, 1995). Haug and co-workers (1994) proposed that following interiorization of aluminium by the cell, metal interactions decrease the accumulation of inositol phosphates, especially IP_3 and derangements of intracellular calcium homeostasis. Moreover, the same authors referred that if present as a phosphate-like fluoro-aluminate, a stimulatory role of aluminium ions is displayed in G protein-coupled transmembrane signalling. Indeed, direct stimulation of G proteins by aluminium tetrafluoride was reported to induce an increase in inositol phosphates formation and $^{45}Ca^{2+}$ efflux (Lo Russo *et al.*, 1997). Furthermore, aluminium at low concentrations (1.25 μM) was found to have a stimulating effect on oligodendrocyte cell cultures by enhancing the production of IP_3, stimulating G protein-linked signal transduction and increasing protein synthesis (Golub *et al.*, 2002). These aluminium activating properties were proposed to be attributable to the aluminium ion acting extracellularly.

Aluminium is also known to interfere with intracellular calcium homeostasis (Shi and Haug, 1992; Haug *et al.*, 1994; Kaur and Gill, 2005). Gandolfi and co-workers (1998) reported that aluminium modifies calcium uptake by the endoplasmic reticulum, accelerates calcium release from mitochondria and strongly inhibits Ca-ATPase activity, with a consequent high-level calcium accumulation inside the cell. In addition, a study on the calcium homeostatic mechanisms in the rat central nervous system revealed that aluminium inhibits Ca-ATPase activity both *in vitro* and *in vivo*, inhibits calcium uptake and affects the biological activity of calcium regulatory proteins, calmodulin and protein

kinase C (Julka and Gill, 1996). These authors suggested that aluminium disrupts calcium homeostasis by interacting with calcium binding sites. It was reported that aluminium ions bind to calmodulin in the presence of calcium ions, leading to an inactive, reversible conformation, instead of its physiological active form. Structural changes of calmodulin, which occur upon aluminium binding, lead to the impairment of protein flexibility and to the loss of its ability to interact with several other proteins, which may decrease or inhibit the regulatory character of calmodulin (Levi *et al.*, 1998). Disruption of neuronal calcium homeostasis was also found after chronic aluminium toxicity in rats (Kaur and Gill, 2005). These authors reported that chronic aluminium administration caused a significant rise in intrasynaptosomal calcium levels, decreased Ca-ATPase activity and increased calcium uptake via voltage-operated calcium channels. An inhibitory effect on calcium uptake and on Ca-ATPase activity was also described in monkey brain, after chronic aluminium exposure (Sarin *et al.*, 1997). Additionally, in primary neuronal cultures, aluminium was found to potentiate glutamate-induced calcium accumulation (Mundy *et al.*, 1997). The aluminium-induced impairment of calcium homeostasis may conduct the cell towards pathways that are detrimental to its survival, such as apoptosis.

I.1.5. Aluminium-induced neuronal apoptosis

Apoptosis, or programmed cell death, is a normal feature in the development of the nervous system and may also play a role in neurodegenerative diseases and aging (Sastry and Rao, 2000). Apoptosis has been suggested to be responsible for the neuronal cell loss observed in many pathological disorders. Mitochondrial changes following cytotoxic stimuli, including the opening of the mitochondrial permeability transition pore (MTP), represent a primary event in apoptotic cell death. The apoptogenic factor, cytochrome *c*, is released, probably due to the MTP opening, from mitochondria into the cytoplasm where it binds to another cytoplasmic factor, Apaf-1, and the complex activates the initiator caspase-9 that in turn activates the effector caspase, caspase-3 (Li *et al.*, 1997). Other regulating proteins, such as the anti-apoptotic Bcl-2 and Bcl-X_L, and the proapoptotic Bax are also involved in controlling and initiating apoptosis (Adams and Cory, 2001). Although, mitochondrial dysfunction has been implicated in neuronal cell death, it seems that the endoplasmic reticulum also has an active role in regulating apoptosis (Savory *et*

al., 2003). In contrast to necrosis, apoptosis is an ordered operation, with characteristic apoptotic morphological changes that include nuclear condensation and fragmentation, DNA damage, cell shrinkage, membrane blebbing, and the formation of membrane-bound apoptotic bodies (Huppertz *et al.*, 1999).

Apoptosis is an active process controlled by genes which can be activated by a variety of stimuli, including oxidative stress and exposure to hormones, toxins and drugs. Indeed, intracisternal injection of aluminium into rabbit brain leads to biochemical changes suggestive of apoptosis (Savory *et al.*, 1999). Apoptotic neuronal loss was observed after intracisternal administration of aluminium complexes to the rabbit brain (Ghribi *et al.*, 2001a). They also revealed that glial cell-derived neurotrophic factor (GDNF) markedly prevents aluminium-induced apoptosis. Aluminium was found to induce stress in both mitochondrial and endoplasmic reticulum, eventually culminating in the activation of caspases and apoptosis (Savory *et al.*, 2003). Cytochrome *c* release from mitochondria and binding to Apaf-1 seems to be the trigger that initiates the aluminium-induced apoptosis cascade (Savory *et al.*, 2003). Ghribi and co-workers (2001a) observed cytochrome *c* release from mitochondria, Bcl-2 down-regulation in both mitochondria and endoplasmic reticulum, Bax translocation into mitochondria, caspase-3 activation and DNA fragmentation, following intracisternal aluminium administration in rabbit brain. In addition, whereas pro-caspase-3 is known to be distributed mainly in the cytoplasm, active caspase-3 was found to be localized mainly in the endoplasmic reticulum following aluminium-induced neurotoxicity in rabbit hippocampus (Ghribi *et al.*, 2002). Aluminium also induced stress in the endoplasmic reticulum in rabbit hippocampus, involving nuclear translocation of *gadd 153*, a transcription factor important in growth arrest and DNA damage induction, and NF-$_K$B which initiates apoptosis (Ghribi *et al.*, 2001b).

The intracisternal injection of aluminium in rat brain also induced apoptosis as assessed by DNA fragmentation and activation of caspase-3 and caspase-12 (Yang *et al.*, 2004). Aluminium-induced apoptosis was also reported in cultured cortical neurons and SAPK/JNK signalling pathways appear to play an important role in the apoptosis induced by aluminium (Fu *et al.*, 2003). Aluminium maltolate was found to cause death of primary cultured rat hippocampal neurons in a time- and dose-dependent manner, and synapse loss was observed (Kawahara *et al.*, 2003). Aluminium-induced apoptosis was also reported in cultured astrocytes which exhibited altered calcium homeostasis (Guo and Liang, 2001).

Moreover, primary cultured astrocytes accumulate aluminium which induces apoptotic features, such as chromatin condensation and fragmentation (Aremu and Meshitsuka, 2005). Apoptosis, including the release of cytochrome *c*, was also verified in the human cell line NT2 after aluminium maltolate treatment, and it appears that the cytochrome *c* release results from an opening of the MTP (Griffioen *et al.*, 2004). In addition, aluminium maltolate induced apoptosis in Neuro-2a cells, a neuroblastoma cell line. Besides apoptotic features such as caspase-3 activation, Bcl-2 down-regulation, Bax up-regulation, and nuclear condensation and fragmentation, the detection of aluminium induced p53 up-regulation was indicative of an important role for p53 signalling in apoptosis induced by aluminium (Johnson *et al.*, 2005).

I.2. PROTEIN PHOSPHORYLATION

I.2.1. Protein phosphorylation as a dynamic process

In eukaryotes, protein phosphorylation is probably the most important regulatory event. Structural and regulatory proteins, namely many enzymes and receptors are switched "on" or "off" (activated or not) by phosphorylation and dephosphorylation. This is a dynamic and reversible process controlled by phosphatases and kinases. Protein phosphatases (PP) are enzymes that catalyze the cleavage of phosphate from serine, threonine and tyrosine residues in proteins; they dephosphorylate proteins, changing their shapes and activities. On the other hand, protein kinases phosphorylate proteins by transferring phosphate from ATP to the protein (Figure I.3). Thus, proper regulation of protein phosphorylation requires the coordinated efforts of both protein phosphatases and kinases. The balance between phosphatase and kinase activities regulates different events such as cell proliferation and metabolism, learning and memory, receptor modulation, neurotransmission, muscle contraction or even gene expression (Walaas and Greengard, 1991; Tapia *et al.*, 1999; Genoux *et al.*, 2002).

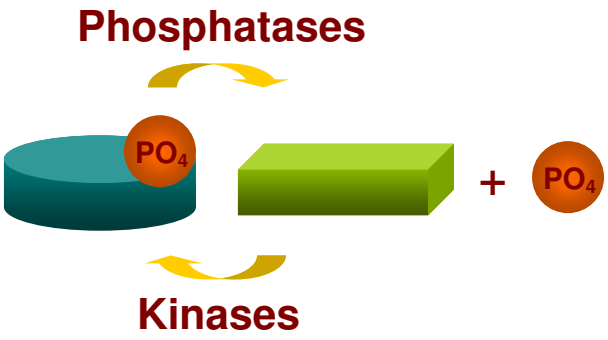

Figure I.3 - Reversible protein phosphorylation. The substrate protein is dephosphorylated by a protein phosphatase and phosphorylated by a protein kinase.

Protein phosphatases and protein kinases are key players in many signal transduction cascades; they are also regulated by a myriad of extracellular and intracellular signals. The human genome encodes a far greater number of serine/threonine protein kinases than of phosphatases. Although, all protein kinases belong to a single family, protein phosphatases are divided into several distinct and unrelated protein/gene families. The serine/threonine-specific protein phosphatases comprise three distinct families. The tyrosine-specific phosphatase family includes the tyrosine-specific phosphatases and the so-called dual specificity phosphatases (capable of dephosphorylating serine, threonine and tyrosine residues). Besides these intracellular phosphatases involved in signal transduction, there are also unrelated non-specific alkaline and acid phosphatases that are usually found either in specialized intracellular compartments or in the extracellular milieu.

I.2.2. Serine/threonine protein phosphatases

Serine/threonine protein phosphatases were initially classified into four classes denominated 1, 2A, 2B and 2C, according to their biochemical characteristics, sensitivity to endogenous inhibitor proteins, dependence upon metal ions and substrate specificity (Ingebritsen and Cohen, 1983). Type-1 phosphatases (PP1) preferentially dephosphorylate the β-subunit of phosphorylase kinase and are inhibited by thermostable protein inhibitor-1 and inhibitor-2 (Cohen, 1989), while type-2 phosphatases (PP2) preferentially dephosphorylate the α-subunit of phosphorylase kinase and are unaffected by inhibitor-1 or inhibitor-2 (Ingebritsen and Cohen, 1983). Type-2 phosphatases are further subdivided into PP2A, PP2B (calcineurin) and PP2C, on the basis of their requirement for divalent

cations. PP2A is active in the absence of divalent cations, while PP2B is dependent upon calcium and stimulated by calmodulin, and PP2C requires magnesium and is okadaic acid-insensitive (Ingebritsen and Cohen, 1983; da Cruz e Silva *et al.*, 1987; Cohen, 1989; Cohen, 1997; Klee *et al.*, 1998). More recently, recombinant DNA and molecular cloning techniques revealed that serine/threonine protein phosphatases are encoded by three gene families, PPP (phosphoprotein phosphatase), PPM (Mg^{2+}-dependent protein phosphatase) and the FCP family; they are defined by distinct amino acid sequences and crystal structures. The PPP family includes the okadaic acid-sensitive phosphatases PP1 and PP2A, and the okadaic acid-insensitive PP2B (Figure I.4), while the PPM family comprises the okadaic acid-insensitive Mg^{2+}-dependent protein phosphatases, including PP2C and pyruvate dehydrogenase phosphatase. The FCP family was more recently recognised through its founding member FCP1 which dephosphorylates the carboxy-terminal domain of RNA polymerase II (Cohen, 2004). This FCP family is also Mg^{2+}-dependent. Another distinct gene family encodes protein tyrosine phosphatases (PTP), which dephosphorylate phosphotyrosine amino acids.

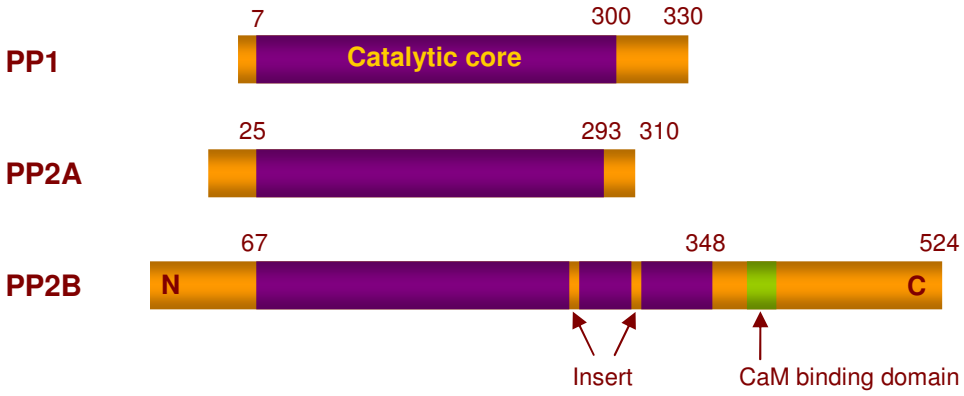

Figure I.4 – Schematic representation of the PPP gene family. These phosphatases contain a common catalytic core domain that is conserved among species. PP1 and PP2A are highly homologous enzymes, differing primarily in their N- and C-terminal domains. PP2B differs in that it contains a Ca^{2+}-calmodulin (CaM) binding site. PP2B contains inserts in the catalytic core that alters the okadaic acid/microcystin toxin binding sites contained in PP1 and PP2A (adapted from da Cruz e Silva, 1998).

Molecular cloning contributed to the protein phosphatases classification in a phylogenetic way, for instance, revealed that PP2A was much more related to PP1 than to

PP2C (Berndt *et al.*, 1987; da Cruz e Silva *et al.*, 1987). PP1, PP2A and PP2B are structurally related to each other, whereas PP2C appears to have a distinct evolutionary background. Furthermore, a group of novel protein phosphatases was discovered, which are more closely related to PP1 and PP2A and include PP4, PP5, PP6 and PP7 (Figure I.5) (Cohen, 1997; Honkanen and Golden, 2002). PP7 is dependent on magnesium but not calmodulin and is activated by calcium. These new protein phosphatases are okadaic acid-sensitive (except PP7) and are expressed in relatively low abundance (Barford *et al.*, 1998), while PP1 and PP2A together have been reported to account for more than 90% of all serine/threonine phosphatase activity in the mammalian cell (Cohen and Cohen, 1989).

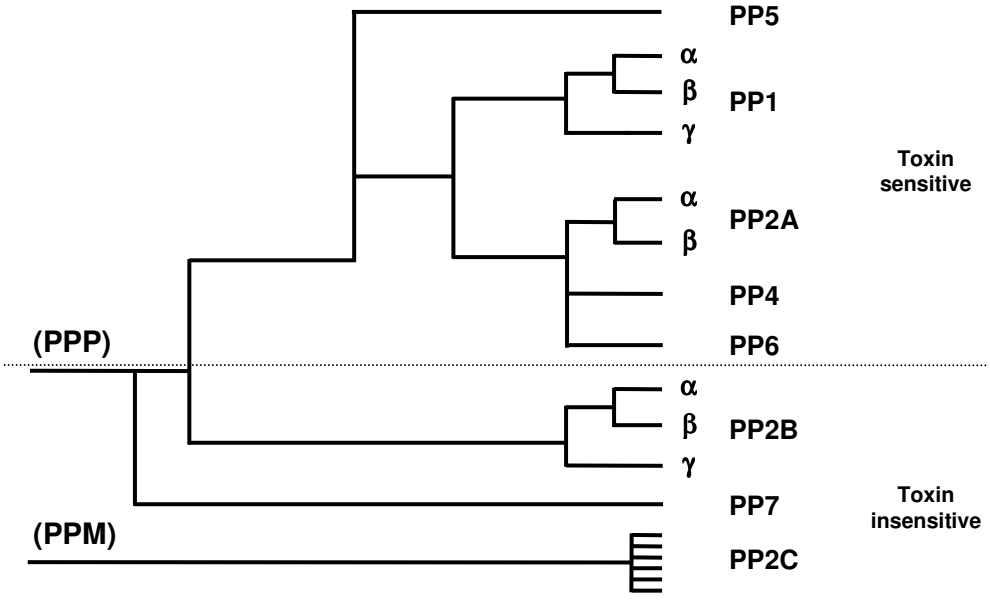

Figure I.5 – Serine/threonine protein phosphatase phylogenic tree. A tree illustrating the similarity between the known PPases based on their primary amino acid sequence. PP1-PP7 belong to a single gene family (PPP) that is structurally distinct from the PP2C family (PPM). The phosphatases above the dashed line are highly sensitive to inhibition by the naturally occurring toxins, okadaic acid, mycrocistin and calyculin A (adapted from Honkanen and Golden, 2002).

I.2.2.1. Protein phosphatase 1

Protein phosphatase 1 (PP1) is one of four major types of serine/threonine phosphatases mediating signalling pathways. PP1 is ubiquitously distributed and has been implicated in the regulation of such diverse biological processes as synaptic plasticity, cell-

cycle progression, protein synthesis, muscle contraction, gene transcription, and glycogen metabolism (Bollen, 2001; Cohen, 2002).

PP1 is highly conserved among species. The identity between human PP1 and yeast reaches approximately 80%, which represents one of the most conserved among all enzymes of these two organisms (Lin *et al.*, 1999). Human PP1 has a molecular mass of 37 kDa (Tung and Cohen, 1984). Three genes are known to encode mammalian type 1 phosphatase catalytic subunits, termed PP1α, PP1β (also called PP1δ) and PP1γ, which demonstrate >90 % homology with each other (Faver *et al.*, 1997). Protein sequence variations among these isoforms are mainly confined to the carboxyl terminus (Sasaki *et al.*, 1990), thought to play a regulatory role in the catalytic activity, such as proteolysis and phosphorylation (Eto *et al.*, 1995). The gene for PP1γ produces two isoforms by alternative splicing, differing only in the extreme carboxyl terminus: $PP1\gamma_1$ is widely expressed in mammalian tissues and $PP1\gamma_2$ is predominantly expressed in testis (da Cruz e Silva *et al.*, 1995b).

The sequence of the catalytic core of PP1 (residues 41-269 of the mammalian α isoform) is nearly identical for all isoforms, and shows a high degree of similarity with the corresponding fragment of the catalytic subunits of PP2A and PP2B (Egloff *et al.*, 1995; Goldberg *et al.*, 1995). This core enzyme (228 residues) has broader substrate specificity than does intact PP1 catalytic subunit (330 residues) and has a severely reduced sensitivity to inhibitory toxins and protein regulators (Connor *et al.*, 1999). The extremities of the catalytic subunit therefore appear to restrict the substrate specificity of the enzyme and are essential for the binding of regulators. Residues 270-296 seems to contribute to the creation of at least two binding sites for regulators (Connor *et al.*, 1999). Yet, intact PP1 catalytic subunit has a rather broad substrate specificity and can dephosphorylate numerous proteins *in vitro* (Bollen and Stalmans, 1992). However, in the cell, the catalytic region interacts with a number of regulatory subunits (Cohen, 1989; Cohen, 1997). The three-dimensional structures of mammalian PP1 catalytic subunits have been defined by crystallography studies (Egloff *et al.*, 1995; Goldberg *et al.*, 1995). The core region of PP1 forms a compact ellipsoidal structure, consisting of a central distorted β-sandwich of 11 β-strands surrounded by 7 α-helices on one side and a subdomain composed of 3 α-helices and a 3-strand β-sheet on the other side. In the central region of the distorted β-sandwich, 3 β-strands connected by 2 α-helices form a β-α-β-α-β motif. This motif is proposed as the

active site of the enzyme where two metal ions bind. The presence of metal ions at the active site suggests that PP1 dephosphorylates its substrates through metal ion-mediated hydrolysis (Egloff *et al.*, 1995; Goldberg *et al.*, 1995).

In contrast to protein serine/threonine kinases whose substrate specificity is in part determined by the presence of sequence motifs flanking the target amino acid, protein phosphatases appear to lack any obvious sequence specificity (Tung *et al.*, 1985). A number of distinct proteins (regulatory subunits) bind the catalytic subunit of PP1 and serve to direct the enzyme toward distinct subcellular locations and/or modify the activity towards specific substrates. Despite the lack of primary structure homology between these regulatory proteins, a PP1 binding motif, RVxF, has been found in the majority of these, including inhibitor-1, DARPP-32 and NIPP1, suggesting that the same domain is required for regulatory binding (Egloff *et al.*, 1997; Zhao and Lee, 1997). Regulatory subunits also modulate the substrate specificity of PP1, thus allowing its activity to be modulated by extracellular signals such as hormones and growth factors (Hubbard and Cohen, 1993). PP1 can dephosphorylate multiple substrates, both *in vitro* and in cell-free assays, with no obvious sequence specificity; this appears to be a consequence of the action of more than thirty regulatory subunits that possess the ability to target the catalytic subunit to specific intracellular locations (Aggen *et al.*, 2000; Bollen, 2001; Gallego and Virshup, 2005; Terrak *et al.*, 2004). Indeed, subcellular targeting gains importance as a crucial regulator of protein phosphatase action (Strack *et al.*, 1997).

A variety of proteins are known to interact with PP1 including its intracellular inhibitor proteins such as inhibitor-1 (I-1), inhibitor-2 (I-2) and DARPP-32. Both I-1 and I-2 are heat stable and are not precipitated by 1% trichloroacetic acid, in contrast to most other proteins. I-1 binds to and inhibits PP1 only after being phosphorylated on Thr-35 by cAMP-dependent protein kinase or cGMP-dependent protein kinase (Hemmings *et al.*, 1984b). Amino acids 9-12 KIQF seem to be crucial for binding and inhibition of PP1 (Egloff *et al.*, 1997). On the other hand, I-2 binds and inhibits PP1 regardless of phosphorylation. I-2 inhibits PP1 via interaction with amino acid Tyr-272 (Zhang *et al.*, 1996). All four PP1 isoforms (α, γ_1, γ_2 and β) are inhibited by proteins I-1 and I-2 (Depaoli Roach *et al.*, 1994; Wera and Hemmings, 1995). DARPP-32 is similar to I-1 in function but derived from a different gene, and is mainly expressed in the brain (Hemmings *et al.*, 1984a). Among several other proteins, neurofilament L (Terry-Lorenzo *et al.*, 2000) and

tau (Liao *et al.*, 1998) where also found to interact with PP1. Liao and co-workers (1998) reported that PP1 is targeted to microtubules by microtubule-associated protein tau and thus is involved in the maintenance of microtubule stability. Moreover, in normal brain, it was shown that PP2A, PP2B and to a lesser extent PP1, are involved in the dephosphorylation of tau (Gong *et al*., 1995).

The tissue expression and cellular or subcellular distribution of PP1 catalytic subunits are not uniform. For instance, in rat salivary glands, only some cell types were reported to react with antibodies specific for PP1γ_1 (Shirakawa *et al.*, 1996). In the heart, where PP1α, PP1β, and PP1γ_1 have been immunologically detected in whole tissue homogenates, the myofibrillar fractions contained mainly PP1α (Chu *et al.*, 1994). In the brain the mRNAs for PP1α, PP1β and PP1γ_1 were found to be particularly abundant in hippocampus and cerebellum (da Cruz e Silva *et al.*, 1995b). At the protein level PP1α and PP1γ_1 were found to be more highly expressed in brain than in peripheral tissues, with the highest levels being measured in the striatum, where they were shown to be relatively enriched in the medium-sized spiny neurons (da Cruz e Silva *et al.*, 1995b). At the electron microscopic level, PP1 immunoreactivity was demonstrated in dendritic spine heads and spine necks and possibly also in postsynaptic density (Ouimet *et al.*, 1995). These authors also observed that most neuronal nuclei were not immunoreactive for PP1γ_1 but were usually strongly immunoreactive for PP1α (Ouimet *et al.*, 1995). Although, PP1γ_1 is known to be widely expressed in mammalian tissues, PP1γ_2 is predominantly expressed in testis (da Cruz e Silva *et al.*, 1995b). Moreover, as well as PP1, the other phosphatases (PP2A, PP2B and PP2C) were also found to be highly expressed in neurons of human brain (Pei *et al.*, 1998).

I.2.2.2. Protein phosphatase 2A

PP2A is a major protein phosphatase in all eukaryotic cells and has a wide range of biological functions. These include the control of cell cycle, organization of cytoskeleton, transcription of immediate early genes, cholesterol and protein biosynthesis. PP2A is a heterotrimeric enzyme composed of a catalytic C subunit, a structural A subunit and a regulatory B subunit. cDNAs encoding two C subunits, two A subunits and over twenty B subunits, belonging to four gene families (B, B', B'', B'''), have been identified. There is

a total lack of sequence similarity between these four gene families, even though the gene products recognize the same or overlapping sites on the A subunit (Ruediger *et al.*, 1992, 1994; Janssens and Goris, 2001). Each B subunit associates with A and C subunits in a mutually exclusive fashion (Kremmer *et al.*, 1997). There are two isoforms of mammalian PP2A C subunits (PP2Aα and PP2Aβ), which share 97% amino acid sequence identity, with the α isoform being approximately ten-fold more abundant than the β isoform (da Cruz e Silva and Cohen, 1987; Stone *et al.*, 1987). Both isoforms are highly conserved in nature, with human and yeast PP2A sharing ~80% identity at the level of their primary amino acid sequences. The structural A subunit also consist of α and β isoforms that are ubiquitously expressed and share 86% sequence identity. The A subunits function as scaffolds that link the C subunit to the different regulatory B subunits (Hemmings *et al.*, 1990; Hendrix *et al.*, 1993). The existence of two C, two A, four B, at least eight B', four B'' and two B''' isoforms, suggests the existence of numerous different PP2A complexes establishing vast possibilities for activation, substrate specificity and sub-cellular localization (Janssens and Goris, 2001). Moreover, it is becoming clear that a major function of the regulatory subunits is to target the PP2A holoenzyme to distinct intracellular locations, signalling complexes and substrates.

PP2A catalytic subunit is regulated by molecular mechanisms that include phosphorylation, carboxylation and stimulation by ceramide. The C subunit can be phosphorylated *in vitro* by tyrosine kinases $p60^{v\text{-}src}$, $p56^{lck}$, epidermal growth factor receptor and insulin receptor (Chen *et al.*, 1992). Phosphorylation occurs on Tyr-307 at the extreme C-terminus of the protein and leads to 90% loss of activity. Dephosphorylation reactivates the phosphatase. The methylation of the α-carboxyl group of the C-terminal Leu-309 of PP2A *in vitro* has only moderate stimulatory effects on phosphatase activity and impairs binding of peptide-specific antibodies to the C-terminus of the PP2A C subunit (Favre *et al.*, 1994). In vitro, ceramide activates the trimeric forms of PP2A that contain either the PR55a (a B subunit) or the 54 kDa protein (a B' subunit). In contrast, PP2A dimeric form and the isolated C subunit are insensitive to ceramide (Dobrowsky *et al.*, 1993). The intracellular PP2A activity is also inhibited by two inhibitor proteins called PP2A I-1 and PP2A I-2 in mammalian tissues (Li *et al.*, 1995; Li *et al.*, 1996). Both are thermostable and not inactivated by 1% trichloroacetic acid. They inhibit PP2A in a noncompetitive manner with the substrate and exhibit apparent inhibition

constant (K_i) values in the nanomolar range (Li *et al.*, 1995). PP2A I-1 appears to inhibit the catalytic subunit directly (Li *et al.*, 1996). PP2A, by dephosphorylating PP1 inhibitors I-1 and DARPP-32, regulates the activity of PP1. Additionally, PP2A I-1 and PP2A I-2 regulate the activity of PP1 by associating with and modifying the substrate specificity of $PP1_C$ in the presence of physiological concentrations of Mn^{2+} (Katayose *et al.*, 2000).

Concerning subcellular distribution, PP2A seems to be mainly cytosolic. However, some PP2A was also detectable in the nucleus (Turowski *et al.*, 1995). Both PP2A catalytic subunit isoforms α and β are ubiquitously expressed, and high levels are found in brain and heart. However, PP2Aα is about 10 times more abundant than PP2Aβ (Khew-Goodall and Hemmings, 1988). Among all tissues tested, PP2A activity was highest in brain extracts (Ingebritsen and Cohen, 1983). The catalytic subunit was found to have a wide regional distribution in brain, with the highest immunoreactivity being present in neurons and particularly enriched in the cytosolic and synaptosolic subcellular fractions (Saitoh *et al.*, 1989).

I.2.2.3. Protein phosphatase 2B

PP2B, also termed calcineurin, is a calcium/calmodulin-activated protein serine/threonine phosphatase. The enzyme consists of two subunits, the catalytic A subunit of 60 kDa (PP2B A) and the regulatory or calcium-binding B subunit of 19 kDa (PP2B B), which makes this the only phosphatase directly regulated by a second messenger (Klee *et al.*, 1988; Cohen, 1989). PP2B is present in nearly all mammalian cells studied. However, it is most highly expressed in the brain. Originally, the term calcineurin referred to the neuronal form of PP2B but, more recently, calcineurin refers to both neuronal and non-neuronal PP2B. The amino acid sequence of PP2B is highly conserved from humans to yeast with over 50% sequence identity (da Cruz e Silva and Cohen, 1989; da Cruz e Silva *et al.*, 1991). Cloning from rat brain indicated an A subunit of 521 amino acids. PP2B catalytic A subunit comprises three isoforms (PP2B Aα, PP2B Aβ and PP2B Aγ) that share >80% identity at the level of their primary amino acid sequence (da Cruz e Silva *et al.*, 1991). Both, Aα and Aβ isoforms are highly expressed in brain, whereas Aγ is testis specific (Ueki *et al.*, 1992). Differential splicing of PP2B Aα generates two transcripts (α_1 and α_2), whereas PP2B Aβ gene is alternatively spliced to give three transcripts β_1, β_2 and

β_3. The PP2B A subunit shows autoinhibition that is relieved by interaction with the B subunit. In contrast to PP1 and PP2A, PP2B is the only PP directly regulated by calcium. The inhibition of B on A is relieved if B binds calcium. This explains why the enzyme is dependent on calcium for activity. Using proteolysis of the autoinhibitory carboxyl terminus of the A subunits generates a calcium-independent form of the enzyme. The B subunit was sequenced at the protein level and found to comprise 168 amino acids, exhibiting a high degree of similarity to calmodulin. Two different B subunit genes are known, the PP2B Bα and the PP2B Bβ. PP2B Bα gives rise to one isoform expressed in many tissues termed PP2B $B\alpha_1$ (170 amino acids) and, by means of a different promoter, leads to another testis-specific isoform termed PP2B $B\alpha_2$ (216 amino acids). PP2B Bβ (179 amino acids) is only expressed in the testis (Ueki *et al.*, 1992; Chang *et al.*, 1994).

PP2B has much narrower *in vitro* substrate specificity than either PP1 or PP2A. This is consistent with its specialized functions in the nervous system, T lymphocytes and other cells. The PP1 inhibitors I-1 and DARPP-32 are excellent *in vitro* and *in vivo* substrates for PP2B, therefore, PP2B can control PP1 activity (Mulkey *et al.*, 1994). PP2B is the target of two clinically important immunosuppressive drugs, cyclosporine and FK506. The complex of each drug with its cognate intracellular receptor, known as an immunophilin, binds to and inhibits the PP2B heterodimer (Liu *et al.*, 1991). A protein called cain, due to its calcineurin inhibitor activity, is highly expressed at the RNA and protein levels in brain, kidney and testis. It is mainly cytosolic. It was speculated that cain may target inactivated PP2B to specific intracellular regions where its release would provide calcium-regulated phosphatase activity to specific signalling pathways (Lai *et al.*, 1998).

I.2.2.4. Protein phosphatase 2C

PP2C is monomeric. In mammalian cells, two PP2C isoforms were known: PP2Cα and PP2Cβ (Tamura *et al.*, 1989; Wenk *et al.*, 1992). PP2Cα is comprised of 382 amino acids. Several isoforms of PP2Cα, namely, $PP2C\alpha_1$, $PP2C\alpha_2$ and $PP2C\alpha_3$, have been reported (Mann *et al.*, 1992), with $PP2C\alpha_1$ being the most abundant isoform. Alternative splicing seems to generate the isoforms $PP2C\beta_1$ and $PP2C\beta_2$. They were cloned from a mouse library and show differences in carboxyl termini and the 3'-untranslated region.

PP2Cβ_1 is expressed in all mouse tissues studied, whereas PP2Cβ_2 is confined to heart and brain where they might subserve special functions (Terasawa *et al.*, 1993). PP2Cβ_1 and PP2Cβ_2 code for 390 and 389 amino acids, respectively. PP2C was originally assumed to be exclusively cytosolic (Shenolikar and Nairn, 1991), but more recent work identified PP2C also in the nucleus of mammalian cells (Das *et al.*, 1996).

I.2.2.5. New protein phosphatases: PP4, PP5, PP6 and PP7

PP4, also called PPX (da Cruz e Silva *et al.*, 1988, Brewis *et al.*, 1993), is expressed highly in testis, however, it was also detected in other tissues. Its structure, like that of PP6, is reminiscent of PP2A. PP4 is comprised of 307 amino acids (rabbit) and is mainly localized in the nucleus, although smaller amounts are also present in the cytosol (Brewis *et al.*, 1993).

PP5 is ubiquitously expressed in human tissues. The calculated molecular mass of the protein is 58 kDa (Chen *et al.*, 1994). PP5 contains an autoinhibitory domain. Polyunsaturated fatty acids can relieve this inhibition (Chen and Cohen, 1997). PP5 was detectable mainly in the nucleus, although some immunoreactivity was also present in the cytosol (Chen *et al.*, 1994).

PP6 is also structurally related to PP2A. PP6 has been identified in all mammalian tissues examined (Mann *et al.*, 1993).

PP7 is comprised of 653 amino acids. PP7 was only detectable in the retina by analysis of RNA from various human tissues (Huang and Honkanen, 1998).

I.2.3. Protein phosphatase inhibitors

Several natural compounds, such as okadaic acid (Tachibana *et al.,* 1981; Takai *et al.*, 1987; Bialojan and Takai, 1988), microcystin (Carmichael *et al.*, 1988; Honkanen *et al.*, 1990), nodularin (Honkanen *et al.*, 1991), tautomycin (Cheng *et al.*, 1987; Takai *et al.*, 2000), fostriecin (Hastie and Cohen, 1998; Walsh *et al.*, 1997), cantharidin (Hastie and Cohen, 1998; Li and Casida, 1992) and calyculin A (Sheppeck *et al.*, 1997; Matsunaga *et al.*, 1997), have been found to inhibit the activity of the PP1-PP6 family of protein

phosphatases. Most of these compounds were originally derived from extracts of natural products and many were initially identified as eukaryotic cell toxins. The organisms that make these inhibitors are biologically diverse, with potent inhibitors produced by dinoflagellates (i.e. okadaic acid), cyanobacteria (i.e. microcystin and nodularin), Streptomyces (i.e. tautomycin and fostriecin), insects (i.e. cantharidin), and calyculin A was identified from a marine sponge extract (Honkanen and Golden, 2002). Table I.1 summarizes the IC_{50} of each compound for each protein phosphatase. As a specific PP1 and PP2A inhibitor, okadaic acid is an excellent tool to evaluate the contribution of those phosphatases in a wide range of systems (e.g. Boudreau and Hoskin, 2005).

Table I.1 - Natural serine/threonine protein phosphatases inhibitors

	Inhibition of Ser/Thr protein phosphatase activity (IC_{50}, nM)					
Compound	**PP1**	**PP2A**	**PP2B**	**PP4**	**PP5**	**PP7**
Okadaic acid	20-50	0.1-0.3	~4 μM	0.1	3.5	>1 μM
Microcystin	0.3-1	<0.1-1	~1 μM	0.15	1	>1 μM
Nodularin	2.4	0.3	>1 μM	ND	ND	>1 μM
Calyculin A	0.4	0.25	>1 μM	0.4	3	>1 μM
Tautomycin	1	10	>1 μM	0.2	10	ND
Cantharidin	450	50	>10 μM	50	3.5 μM	ND
Fostriecin	45-58 μM	1.5-5.5	>100 μM	3	70 μM	ND

(Adapted from Honkanen and Golden, 2002)

Besides natural toxins, the serine/threonine protein phosphatases also have endogenous protein inhibitors. PP1 specific inhibitors include inhibitor 1 (I-1) and inhibitor 2 (I-2) (Huang and Glinsmann, 1976), both are cytosolic heat-stable proteins with molecular mass of 18.7 kDa and 22.9 kDa, respectively. I-1 and I-2 inhibit PP1 activity with inhibition constants (K_i) of 1.6 nM and 3.1 nM, respectively. Other protein inhibitors of PP1 are dopamine-and-cAMP-regulated phosphoprotein of Mr 32,000 Da (DARPP-32) (Walaas *et al.*, 1983), nuclear inhibitor of PP1 (NIPP-1) (Beullens *et al.*, 1992), C-kinase activated phosphatase inhibitor of Mr 17,000 Da (CPI17) (Eto *et al.*, 1995) and ribosomal inhibitor of PP1 (RIPP-1) (Beullens *et al.*, 1996). I-1 and DARPP-32 need to be phosphorylated to inhibit PP1. However, I-2 and NIPP-1 are more potent phosphatase inhibitors in their dephosphorylated state. PP2A specific protein inhibitors include inhibitor 1 of PP2A and inhibitor 2 of PP2A. Both are thermostable endogenous proteins

of 30 kDa and 39 kDa, which specifically inhibit PP2A with K_i of 30 nM and 2 nM, respectively (Li *et al.*, 1995; Li *et al.*, 1996). As described above PP2B also has an endogenous inhibitor identified as cain (Lai *et al.*, 1998) with 240 kDa that inhibits PP2B with a K_i = 0.44 μM (Oliver and Shenolikar, 1998). The specificity of these inhibitors for a given phosphatase makes them key tools in the study of phosphorylation dependent processes.

I.2.4. Abnormal phosphorylation of proteins

Abnormal protein phosphorylation has been associated with various disorders, including cancer, diabetes, and several neurodegenerative disorders such as ALS, Parkinson's disease, Huntington's disease and AD (Grundke-Iqbal *et al*., 1986b; Goedert *et al*., 1997; Sridhar *et al*., 2000). Hyperphosphorylated neurofilaments and tau proteins are known to be associated with various neurodegenerative diseases (Wang *et al.*, 2001; Wakayama *et al.*, 1993a; Grundke-Iqbal *et al*., 1986b).

I.2.4.1. Neurofilaments

Neurofilaments (NF), the neuron-specific intermediate filaments, are a major cytoskeletal component in neurons. In combination with other cytoskeletal proteins, NF of ~10 nm diameter cross-link with one another and form a three-dimensional network in the axon, defining the calibre and shape of the axon and providing an anchor for other constituents of the cytoplasm. NF are composed structurally of two unique portions: a central shaft 10-12 nm in diameter, called core filament, and peripheral strands 4-5 nm in diameter deriving from the core filament, called projections. Those projections may be connected with projections from other filaments forming cross-bridges. NF are co-assembled from three subunits in mammals, referred to as NF high (NF-H), medium (NF-M) and low (NF-L) molecular weight subunit, with apparent molecular masses of approximately 180-200 kDa, 140-160 kDa and 68-70 kDa, respectively (Lee and Cleveland, 1996) (Figure I.6). The three NF subunits share a characteristic ~310-amino-acid α-helical central domain containing a hydrophobic heptad repeat essential for

assembly. Flanking this central rod are globular head and tail segments, which are markedly divergent in length and sequence among the three subunits. Although the head domains of NF subunits do not share obvious amino acid sequence homologies, they are rich in serine and threonine residues. Phosphorylation and *O*-glycosylation of these residues are believed to be important for *in vivo* regulation of NF assembly. The most distinct features of the NF subunits are the carboxy-terminal (C-terminal) tail domains. For NF-L, this region is highly acidic, with many glutamic acid residues comprising a segment sometimes referred to as the segment E. NF-M has a longer tail domain, also contains E segments as well as segments rich in glutamic acid and lysines. The NF-H tail is unique since it possesses multiple repeats of lysine-serine-proline (KSP). Although the number and the distribution of repeats differ among vertebrates, the serines in the KSP domains are extensively phosphorylated in axons (Lee and Cleveland, 1996; Gotow, 2000). NF-M also contains a few KSP motifs. Their number and position are not conserved, but they are phosphorylated in axons (Xu *et al.*, 1992b). The two major types of KSP motifs in the NF-H tail domain are KSPXKX and KSPXXX (Chin and Liem, 1990). NF-L is less phosphorylated and the phosphorylation sites are in the amino-terminal (N-terminal) of the protein (Hashimoto *et al.*, 2000) (Figure I.6). Phosphorylation of the head domain of NF-L may play an important role in NF dynamics (Nixon, 1993; Hashimoto *et al.*, 2000).

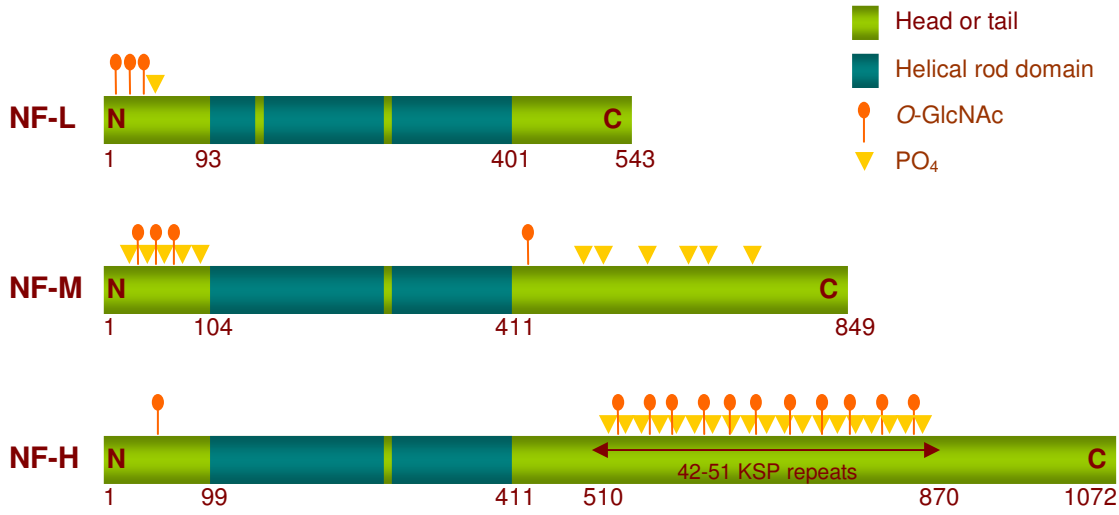

Figure I.6 – Schematic diagram of mammalian NF-L, NF-M and NF-H neurofilament subunits. The numbers below correspond to amino acids from the murine neurofilament subunits. Variable head and tail domains flank the conserved α-helical rod domains. Known or predicted glycosylation sites are indicated, as well as known sites of phosphorylation (adapted from Lee and Cleveland, 1996).

All three NF subunits are integral components of NF. The NF assembly requires the heteropolymerization of NF-L with either NF-M or NF-H in rodents (Lee *et al.*, 1993; Jacomy *et al.*, 1999), although, human NF-L alone can form homopolymers when expressed in a mammalian cell (Carter *et al.*, 1998). The core filaments are built up by both N-terminal head and central conserved helical domains of all three NF subunit proteins, whereas the projections are formed from C-terminal tail domains of both NF-M and NF-H (Gotow *et al.*, 1992). Following synthesis, NF are assembled in the cell body and, it seems that for efficient NF protein transport *in vivo*, the formation of hetero-oligomers is required (Yuan *et al.*, 2003). Assembled NF move into and through axons via slow axonal transport, the rate for which varies between 0.1-1 mm/day. The velocity depends on: the group of neurons, the age of the animal, and the location along the nerve (Lee and Cleveland, 1996). However, it has been reported that NF polymers move along the axon in a rapid, intermittent, bidirectional and highly asynchronous manner (Brown, 2000; Wang *et al.*, 2000). The NF moved at peak rates of up to 3 μm/s, or did not move at all during the observation period. Thus, the peak rate of movement in slow axonal transport may be fast, but the overall rate is slow because the rapid movements are interrupted by prolonged pauses (Wang and Brown, 2001; Ackerley *et al.*, 2003; Brown *et al.*, 2005). During axonal transport NF are extensively phosphorylated on the C-terminal tail of NF-H and NF-M (Nixon, 1993; Nixon *et al.*, 1994). The extent of phosphorylation of the KSP repeat domain promotes the slowing of NF transport rate (Nixon *et al.*, 1994; Shea *et al.*, 2003), apparently this is due to increased pausing in NF movement (Ackerley *et al.*, 2003). Indeed, hypophosphorylated NF are transported more quickly than extensively phosphorylated ones (Jung *et al.*, 2000). Additionally, using knockout mice for the NF-M and NF-H genes, the absence of the NF-M protein resulted in an accelerated rate of slow axonal transport of NF in sciatic nerves (Jacomy *et al.*, 1999). However, a controversy arose when Rao *et al.* (2002) reported, and Yuan *et al.* (2006) confirmed, that NF transport rate *in vivo* is not altered by deleting the extensively phosphorylated NF-H tail domain. Shea *et al.* (2003) proposed that deletion of the NF-H C-terminal region accelerates the transport of a subpopulation of NF, using the same NF-H tail-depleted mutant mice used by Rao and Yuan and co-workers. In fact, it was reported that cdk5, a kinase responsible for the NF C-terminus phosphorylation, regulates axonal transport and phosphorylates NF in cultured neurons (Shea *et al.*, 2004). The authors demonstrated that

overexpression of cdk5 increases NF phosphorylation and inhibits NF axonal transport; consequently phosphorylated-NF "bundles" were found in perikarya. NF transport into and along axons requires microtubules (Helfand *et al.*, 2004; Francis *et al.*, 2005) and microtubule-associated motor proteins, such as kinesin and cytoplasmic dynein, which mediate the translocation of NF along microtubules (Yabe *et al.*, 1999; Shah *et al.*, 2000; Wagner *et al.*, 2004; He *et al.*, 2005). Kinesin mediates anterograde (from cell body to axon tip) NF axonal transport (Yabe *et al.*, 1999). Both NF-H and NF-M regulate the association of NF with kinesin and the phosphorylation of NF-H dissociates NF from kinesin contributing to the slowing of NF axonal transport (Jung *et al.*, 2005). On the other hand, dynein mediates retrograde NF transport within axons (Shah *et al.*, 2000; Wagner *et al.*, 2004; He *et al.*, 2005). Dynein also mediates anterograde delivery of NF from perikarya into axons, and C-terminal NF phosphorylation promotes motor association (Motil *et al.*, 2006). NF transport also appears to depend on actin and myosin (Helfand *et al.*, 2004; Jung *et al.*, 2004). NF are intrinsic determinants of radial growth of axons and phosphorylation of NF-H and NF-M are likely to play a critical role in organizing NF and in their ability to mediate control of caliber (Lee and Cleveland, 1996). Indeed, it was reported that the C-terminal domain of NF-M is essential for the radial growth and cytoskeletal architecture of axons (Rao *et al.*, 2003). Still, the deletion of the tail domain of NF-H seems not to alter the axonal caliber (Rao *et al.*, 2002). Moreover, it was reported that the absence of the NF-M subunit resulted in a two- to threefold reduction in the caliber of large myelinated axons, whereas the lack of NF-H subunits had little effect on the radial growth of motor axons (Jacomy *et al.*, 1999). Another posttranslational modification of the NF subunits concerns the addition of *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) moieties (Dong *et al.*, 1993; Dong *et al.*, 1996). Finally, NF are degraded by proteases upon arrival at the nerve terminals. NF can be degraded by a variety of proteases, particularly those activated by calcium (Greenwood *et al.*, 1993). Phosphorylation seems to protect NF against proteolysis (Goldstein *et al.*, 1987). Thus, C-terminal tail domain phosphorylation regulates NF axonal transport, dynamics and proteolysis (Nixon, 1998; Jung and Shea, 1999; Ackerley *et al.*, 2003). Additionally, tail domain phosphorylation may control inter-filament distance and, indirectly, axon diameter through electrostatic repulsion between the side arms of adjacent NF (Nixon *et al.*, 1994; Lee and Cleveland, 1996; Rao *et al.*, 2003). In contrast, phosphorylation of the N-terminal head domain

appears to prevent premature polymerization of NF subunits in the cell body (Sihag and Nixon, 1990; Sihag and Nixon, 1991).

Under normal conditions the phosphorylation states of the two larger NF protein subunits, specially NF-H, are different between the axonal and somatodendritic compartments. That is, phosphorylated NF appear to be localized mainly in the axon and absent from the cell body, whereas the nonphosphorylated forms are found in the perikarya and dendrites (Sternberger and Sternberger, 1983; Gotow *et al.*, 1995), suggesting the importance of NF phosphorylation in its transport and interaction with other cytoskeletal proteins. Corresponding to the difference in phosphorylation level between these two compartments of the neuron, there is a morphological difference in NF organization, i.e., NF are much denser and arranged into parallel bundles through well-developed cross-bridges in the axons, whereas they are sparse and aligned irregularly with less-developed cross-bridges in the perikarya and dendrites. This features indicates that phosphorylation of NF-H is necessary for the interaction of projections with other projections to form regular cross-bridges, while dephosphorylation is necessary for the interaction of projection with other structures such as microtubules or membrane-bound organelles (Hisanaga *et al.*, 1991; Gotow *et al.*, 1995). Indeed, the interaction of NF-H with microtubules was reported to be accelerated when NF-H was dephosphorylated *in vitro* (Hisanaga *et al.*, 1991).

Since, NF are confined to the nervous system, they might be one of the best markers reflecting neuronal pathogenic changes seen in some neurological disorders. In fact, neuronal accumulation of abnormally phosphorylated NF has been seen in several neurodegenerative diseases, namely: in motor neurons in ALS, in NFT in AD, and in aluminium-induced encephalopathies. The NF accumulate in the neuronal perikaryal compartment and their NF-H tends to be hyperphosphorylated. The accumulation of hyperphosphorylated NF in perikarya might be associated with axonal dysfunction or degeneration, resulting in neuronal death (Gotow, 2000). The accumulation of phosphorylated NF also reflects an anomaly of NF processing, inefficient degradation by proteases, enhanced and aberrant phosphorylation and protein misfolding. All of these features are also noted in the normal aging brain, without manifestation of dementia. Insidious accumulation of these aberrant misfolded proteins in large quantities interferes with synaptic transmission in critical topographic brain areas and may manifest as motor

neuron disease or AD-type dementia (Gupta *et al.*, 2005). In AD brain, NF in addition to tau are hyperphosphorylated and accumulated. In fact, the levels of all three NF subunits have been found to be markedly increased and at least NF-H and NF-M appear to be significantly hyperphosphorylated at several sites (Wang *et al.*, 2001; Hu *et al.*, 2002).

Several evidence points to the involvement of protein phosphatases in the regulation of the phosphorylation of neurofilaments. Veerana *et al.* (1995) suggested the involvement of PP2A in the regulation of the phosphorylation state of KSPXKX motifs in NF-H. PP2A as well as PP1 were found to be associated with and dephosphorylate all three NF subunits *in vitro* (Strack *et al.*, 1997). Wang *et al.* (2001) also reported that treatment of human neuroblastoma SY5Y cells with okadaic acid, an inhibitor of PP2A and PP1, also results in the phosphorylation and accumulation of NF-H and NF-M subunits, as seen in AD. Similarly, Gong *et al.* (2003) reported that inhibition of PP2A induces an increase in the phosphorylation of NF-H and NF-M subunits and a general accumulation of NF throughout the whole neuron, using metabolically active rat brain slices. In addition, NF-L was reported to be a PP1 binding protein (Terry-Lorenzo *et al.*, 2000). Moreover, it was reported that a form of PP2A associates with NF and dephosphorylates NF-L phosphorylated by exogenous protein kinase A, a kinase which phosphorylates the N-terminal (Saito *et al.*, 1995). On the other hand, the cyclin-dependent kinase-5 (cdk5) family could be responsible for the phosphorylation of KSP sequences in the NF-H and NF-M tail domain (Shetty *et al.*, 1993; Veerana *et al.*, 1995; Ackerley *et al.*, 2003). It was also reported that the stress activated protein kinase-1b phosphorylates NF-H tail (Brownlees *et al.*, 2000). Protein kinase A, protein kinase C and CaM kinase II have all been reported to be responsible for the phosphorylation of specific sites in the N-terminal head domain of NF-L and NF-M (Sihag and Nixon, 1990; Sihag and Nixon, 1991; Carter *et al.*, 1996; Sihag *et al.*, 1999).

I.2.4.2. Tau protein

Microtubule-associated protein τ (tau) is essential in maintaining the microtubular integrity of the cell by promoting the assembly and stabilizing the microtubules in neurons. The state of phosphorylation of tau interferes with its normal function to bind with

microtubules. Non-phosphorylated tau associates with microtubules and hyperphosphorylated tau dissociates from microtubules (Drechsel *et al.*, 1992).

In AD and other tauopathies, tau protein, the major component of neurofibrillary tangles (NFT), is abnormally hyperphosphorylated at several serine/threonine sites, aggregated into paired helical filaments (PHF) in affected neurons, and does not bind to microtubules or stimulate microtubule assembly (Figure I.7).

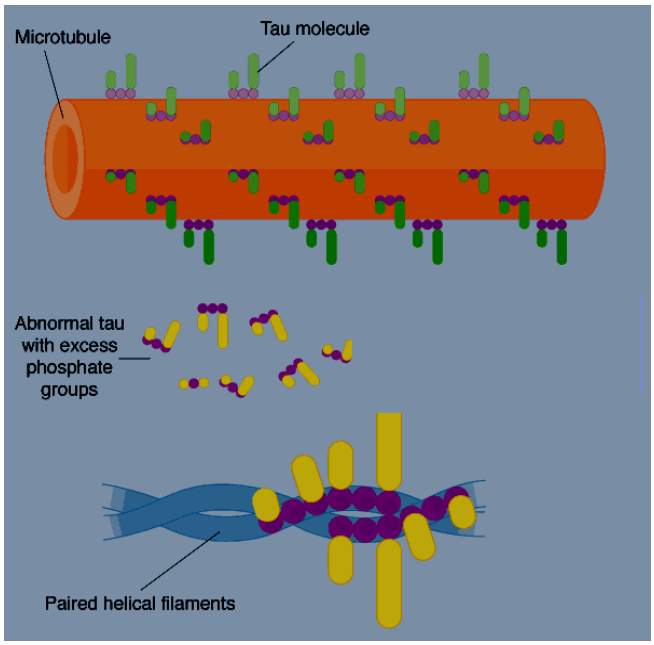

Figure I.7 – Paired helical filament (PHF) formation scheme. Tau protein in its normal state helps microtubule to assemble, however when it is hyperphosphorylated, it aggregates into PHF (adapted from www.abpi.org.uk/publications/publication_details/targetAlzheimers/industry_d.asp).

Abnormal hyperphosphorylation of tau is believed to lead to the neurofibrillary degeneration observed in those diseases. Tau associated with PHF is insoluble, displays retarded mobility on SDS-PAGE, besides being abnormally hyperphosphorylated (Grundke-Iqbal *et al.*, 1986b; Lee *et al.*, 1991). The abnormal phosphorylation of tau is essential for the self-assembly of tau into tangles of PHF, since this assembly is abolished by its dephosphorylation (Alonso *et al.*, 2001). Moreover, the switch of tau protein to an AD-like state includes the phosphorylation of two serine-proline motifs (Ser 199/202) upstream of the microtubule binding region (Biernat *et al.*, 1992).

The molecular mechanism leading to the abnormal hyperphosphorylation of tau in AD is still not well understood. Tau phosphorylation is catalysed by tau protein kinases and reversed by tau protein phosphatases. Among the protein kinases that have been implicated in the abnormal phosphorylation of tau associated with the formation of PHF

are glycogen synthase kinase-3 (Ishiguro *et al.*, 1993), neuronal proline-directed protein kinase (Paudel *et al.*, 1993), mitogen-activated protein kinase (Drewes *et al.*, 1992), calcium/calmodulin-dependent protein kinase II (Steiner *et al.*, 1990), casein kinase I (Singh *et al.*, 1995), cAMP-dependent protein kinase (Jicha *et al.*, 1999) and cyclin-dependent protein kinase-5 (Noble *et al.*, 2003).

More than 30 phosphorylation sites have been identified in the hyperphosphorylated tau isolated from AD brain and all are either on serine or threonine residues. Thus, serine/threonine protein phosphatases (PP) are expected to be involved in the regulation of the phosphorylation of tau in the AD brain. In normal brain, it was shown that PP2A, PP2B and, to a lesser extent, PP1 are involved in the dephosphorylation of tau (Gong *et al*., 1995). On the other hand, by using the abnormally hyperphosphorylated tau protein isolated from AD brain as a substrate, it was found that PP2A, PP2B, and to a lesser extent PP1, but not PP2C, can efficiently dephosphorylate tau *in vitro* (Wang *et al.*, 1995; Gong *et al.*, 1994a, b ,c; Rahman *et al.*, 2006). PP2A has been reported to regulate tau phosphorylation *in vivo* (Gong *et al.*, 2000). PP2B isolated from human brain was found to dephosphorylate serine residues of the Alzheimer disease abnormally hyperphosphorylated tau (Rahman *et al.*, 2006). In addition, PP5 was also reported to be responsible for the dephosphorylation of tau protein (Liu *et al.*, 2005a). PP1, PP2A, PP2B and PP5 all dephosphorylate tau at serine or threonine sites, but each protein phosphatase catalyses the dephosphorylation of tau at different sites with different efficiencies. The relative contributions of PP2A, PP1, PP5 and PP2B to the regulation of tau phosphorylation have been estimated to be ~71%, ~11%, ~10% and ~7%, respectively (Liu *et al.*, 2005b). Moreover, it has been reported that the expression and/or activities of PP1, PP2A, PP2B and PP5 are decreased ~20-30% in the affected areas of AD brain (Gong *et al.*, 1993, 1995; Lian *et al.*, 2001; Liu *et al.*, 2005a). Thus, the abnormal phosphorylation of tau in AD has been suggested to be the result of a deficiency of the protein phosphatase system (Trojanowski and Lee, 1995). Namely, a down-regulation of tau phosphatases in AD brain might underlie the abnormal hyperphosphorylation of tau (Liu *et al.*, 2005b).

PP2A is thought to be the major tau phosphatase that regulates its phosphorylation at multiple sites in human brain. The abnormal hyperphosphorylation of tau may be partially due to a down-regulation of PP2A activity in AD brain. Indeed, the inhibition of PP2A by okadaic acid induced hyperphosphorylation and accumulation of tau (Gong *et al*.,

2000). Also, *in vivo* studies using rats injected with PP1 and PP2A inhibitors, tau phosphorylation was achieved (Janke *et al*., 1998). Moreover, Tanimukai and co-workers found a significant increase in the neocortical levels of I1 and I2, endogenous inhibitors of PP2A, in AD, as compared to control cases, suggesting the possible contribution or involvement of I1 and I2 in the abnormal hyperphosphorylation of tau in AD (Tanimukai *et al.*, 2005). Furthermore, a reduction of PP2A activity in AD brains might keep the PP1 inhibitors, I1 and DARPP32, up-regulated and thereby result in decreased PP1 activity (Iqbal *et al.*, 2005). Using metabolically active rat forebrain slices, it was reported that a decrease in PP2A activity leads to abnormal hyperphosphorylation of tau at Ser 198/199/202, Ser 396/404 and Ser 422, and PP1 up regulates the phosphorylation of tau at those residues (Bennecib *et al.*, 2000). PP2A also regulates the activities of several tau kinases in brain. Additionally, the decrease in the PP2A and PP1 activities in the brain leads to hyperphosphorylation of tau at Ser 262/356, not only by inhibition of its dephosphorylation but also by promoting the calcium/calmodulin-dependent protein kinase II activity in rat forebrain cytosol (Bennecib *et al.*, 2001). Pei *et al.* (2003) reported that okadaic acid-induced inhibition of PP2A produces activation of mitogen-activated protein kinases ERK1/2, MEK1/2 and p70 S6, through dephosphorylation of serine/threonine residues of those kinases, similar to that in AD, and suggested that in AD brain the decrease in PP2A activity could have caused the activation of ERK1/2, MEK1/2 and p70 S6 kinase, and the abnormal phosphorylation of tau both via an increase in its phosphorylation and a decrease in its dephosphorylation. Thus, the hyperphosphorylation of tau in AD may be due to a protein phosphorylation/dephosphorylation imbalance produced by a decrease in the activity of protein phosphatase and increase in the activities of tau kinases which are directly or indirectly regulated by protein phosphatases (Iqbal *et al.*, 1998, 2002, 2005).

I.3. ALUMINIUM CONTRIBUTION TO ABNORMAL PROTEIN PHOSPHORYLATION

Aluminium has not only been implicated in several neurological and other disorders, but is also known to induce the hyperphosphorylation of neurofilaments and tau proteins (Shea *et al.*, 1992b; Shin *et al.*, 1994; Singer *et al.*, 1997), and to promote the aggregation of proteins such as hyperphosphorylated tau (Shin *et al.*, 1994; Savory *et al.*, 1998; Shin, 2001) and Abeta (Exley, 1993; Kawahara *et al.*, 1994; Ricchelli *et al.*, 2005).

I.3.1. Aluminium and tau

Aluminium has long been a target of research concerning its role as an environmental risk factor in the aetiology of AD. High levels of aluminium were found in AD patients brains (Kruck and McLachlan, 1988) and this metal has been correlated with both hallmark lesions of AD, being found co-localised with silicon in the central region of senile plaque cores in the cortex of AD patients (Candy *et al.*, 1986; Tokutake *et al*., 1995) and accumulating in association with NFT in affected neurons (Perl, 1988; Shin *et al*., 1995). It has been suggested that aluminium binds selectively to PHF tau (Shin *et al.*, 1994; Savory and Garruto, 1998) and induces the aggregation of hyperphosphorylated tau (Shin *et al.*, 1994; Singer *et al.*, 1997; Savory *et al*., 1998). Abnormally phosphorylated tau has been found in aluminium-induced neurofilamentous aggregates in rabbits, together with abnormally phosphorylated neurofilament protein (Singer *et al.*, 1997). It was observed that purified recombinant human tau protein incubated with aluminium salts induces noncovalent tau aggregation into NFT-like bundles and this tau coalescence can be reversed by addition of EDTA. However, when incubated with a nucleotide triphosphate such as ATP, aluminium catalyzes a covalent linkage that results in the incorporation of phosphate into the tau protein via an *O*-linkage to the alpha-phosphate (Abdel-Ghany *et al.*, 1993). These reactions occur at aluminium concentrations similar to those found in AD brains suggesting a physiological significance *in vivo* in neurodegenerating brain tissue. Aluminium-induced aggregation of hyperphosphorylated tau seems to be by interacting directly with the phosphorylated epitopes of tau, in AD brains (Zatta, 1995;

Murayama *et al*., 1999; Shin, 2001). It was also reported that the binding of aluminium to PHF tau besides inducing its aggregation also retards its proteolysis (Shin *et al.*, 1994). Moreover, aluminium is known to bind avidly to phosphate groups in proteins, and this binding can modify the conformation of the proteins (Martin, 1986; Birchall and Chappell, 1988). It was suggested that aluminium induces aggregation of brain cytoskeletal proteins by forming very stable bridges between phosphate groups of the hyperphosphorylated tau elements (Zatta, 1995). Moreover, the accumulation of aluminium in the neurofibrillary lesions is attributable to the presence of PHF tau that provides binding sites for aluminium at its phosphorylated epitopes, including Ser 202, Ser 396, Ser 404 and Ser 422, and the biological consequence of the aluminium interaction involves formation of PHF tau aggregates that persist in the affected neurons (Shin *et al.*, 1994; Murayama *et al.*, 1999; Shin, 2001). Iron was also shown to accumulate in neurons with NFT, and in its ferric form (Fe^{3+}) has the ability, similar to that of aluminium (Al^{3+}), to bind with tau and to induce its aggregation *in vitro* (Yamamoto *et al*., 2002). Furthermore, it has been recently reported that a novel trivalent cation chelator dissociates binding of aluminium and iron associated with hyperphosphorylated tau of AD (Shin *et al*., 2003).

Besides, having been implicated in the aggregation of hyperphosphorylated tau into PHF leading to the formation of NFT, aluminium was also suggested to contribute to tau aggregation by altering its phosphorylation state. The dephosphorylation of tau by PP2A was reported to be inhibited by aluminium (Yamamoto *et al.*, 1990). Aluminium was found to inhibit the phosphorylation of tau by neuronal cdc2-like kinase and dephosphorylation of phosphorylated tau by PP2B (Li *et al.*, 1998). Li and co-workers (1998) also observed that phosphorylated tau is more sensitive to aluminium-induced aggregation than nonphosphorylated tau and suggested that phosphorylation sensitizes tau to aluminium and phosphorylated tau transforms irreversibly into a phosphatase and protease resistant aggregate in the presence of this metal ion (Li *et al.*, 1998).

I.3.2. Aluminium and Abeta

Beta-amyloid (Abeta), the major component of senile plaques in AD brain, has an intrinsic tendency to form insoluble aggregates. Abeta is a 39-43 amino acid long peptide derived from a larger transmembrane protein, the amyloid precursor protein (APP). It has

been suggested that aluminium induces the aggregation, deposition and toxicity of Abeta (Tokutake *et al.*, 1995; Evans and Harrington, 1998; Pratico *et al.*, 2002). Exley *et al.* (1993) first demonstrated by circular dichroism spectroscopy that aluminium causes conformational changes of Abeta (1-40). In physiological buffers, Ca, Co, Cu, Mn, Mg, Na or K had no effect on the rate of Abeta aggregation. In contrast, Al, Fe and Zn under the same conditions strongly promoted Abeta aggregation (Mantyh *et al.*, 1993). Other authors also reported that aluminium promotes the aggregation of Abeta (Kawahara *et al.*, 1994; Exley, 2005; Ricchelli *et al.*, 2005). Moreover, long-term exposure to aluminium promoted the aggregation of Abeta on cultured cortical neurons and enhanced its neurotoxicity (Kuroda *et al.*, 1995). Chronic dietary administration of aluminium was found to increase Abeta levels and accelerate plaque deposition in APP transgenic mice, a model of AD-like amyloidosis (Pratico *et al.*, 2002), an *in vivo* evidence for Abeta accumulation induced by aluminium. Ricchelli and co-workers (2005) studied the structural effects induced by aluminium on different Abeta fragments and found that aluminium caused peptide enrichment in beta sheet structure and formation of solvent-exposed hydrophobic clusters. These intermediates evolved to polymeric aggregates which organized in fibrillar forms in the case of the aluminium Abeta (1-42) complex. However, Nakagawa *et al.* (2005) reported that aluminium chloride does not facilitate deposition of human synthetic Abeta (1-42) peptide in the rat ventricular system of a short-term infusion model. Indeed, synthetic Abeta (1-42) aggregates with aluminium chloride almost disappear from the rat ventricular system by 28 days post-infusion. However, Clauberg and Joshi (1993) suggested that aluminium accumulated in AD brain accelerates the generation of Abeta due to the faulty proteolysis of normal APP. Therefore, aluminium by enhancing the processing of APP, leads to the accumulation of Abeta and plaque formation. Furthermore, it was reported that aluminium increases the levels of Abeta in neuroblastoma but not in glioma cells (Campbell *et al.*, 2000). Aluminium-aggregated Abeta tightly binds to the surface of cultured neurons and forms fibrillar deposits, while zinc-aggregated Abeta was rarely observed on the surface of cultured neurons (Kawahara *et al.*, 2001). The authors suggested that aluminium-aggregated Abeta has a strong affinity to membrane surface and is poorly degraded by proteases.

The catabolism of Abeta may be important to its accumulation in the brain in both early and late-onset AD. The serine protease plasmin is one of a suite of proteases

implicated in AD. It is a promoter of alpha-cleavage of APP and degrades Abeta *in vitro*. Korchazhkina *et al.* (2002) demonstrated that the cleavage of the amyloidogenic Abeta (25-35) by plasmin produces the non-amyloidogenic fragment Abeta (29-35). They also found that the degradation of Abeta (25-35) by the serine protease plasmin is inhibited by aluminium. On the other hand, aluminium also modulates APP. Accumulation of APP in damaged neuronal processes and microglia was observed following intracerebral administration of aluminium salts in rat brain (Shigematsu and McGeer, 1992). Aluminium was also found to promote aggregation of human recombinant APP *in vitro*, in a dose-dependent manner (Chong and Suh, 1995). In addition, Sinclair and Pennington (1996) presented aluminium-induced changes in APP mRNA expression. Furthermore, it is known that protein phosphorylation modulates APP processing (da Cruz e Silva *et al*., 1993), more specifically PP1 was shown to be involved in this process (da Cruz e Silva *et al*., 1995a; da Cruz e Silva and da Cruz e Silva, 2003). Abeta-formation in rat brains, after *in vivo* inhibition of PP1 and PP2A was also reported (Arendt *et al*., 1998). The decrease in the phosphatase activities in AD might also be involved in the formation of Abeta by augmenting the amyloidogenic pathway processing of APP (Gong *et al*., 1993).

I.3.3. Aluminium and neurofilaments

It is known that the accumulation of NF in the perikarya and dendrites of neurons occurs when the rabbit brain is treated with aluminium (Klatzo *et al.*, 1965; Crapper *et al.*, 1973; Erasmus *et al.*, 1993; Strong *et al.*, 1996; Savory *et al.*, 1999; He and Strong, 2000; Forbes *et al.*, 2002). In fact, NF began to accumulate a few hours after the intracisternal injection of aluminium (Bugiani and Ghetti, 1982). Those rabbits developed neurological symptoms with paralysis of their skeletal muscles and died with tetanic spasm just over 10 days after the administration of aluminium. Perikaryal NF accumulation was observed in the pyramidal neurons of the cerebral cortex and hippocampus, motor neurons of spinal cord, ganglion cells, bipolar cells of retina, and Purkinje cells of cerebellum (Erasmus *et al.*, 1993). Compared with axonal NF, the NF accumulated in the perikarya were found to be densely compact, composed of less-developed cross-bridges and more irregularly aligned core filaments. Additionally, in contrast to the phosphorylated NF-H localization under normal conditions (abundant in axons and absent in the cell body) in aluminium

treated rabbits the phosphorylated NF-H were detected in perikarya, although they were less phosphorylated than in axons (Troncoso *et al.*, 1986; Shea *et al.*, 1989; Gotow *et al.*, 1995; Gotow, 2000). The difference in the density is probably the result of the presence of less developed cross-bridges. Irregularly aligned core filaments, as well as less developed cross-bridges may be due to the lower phosphorylation level of NF-H. Highly phosphorylated NF-H are necessary for NF to form well developed cross-bridges and the resultant regularly aligned core filaments, as occurs in axons. The increase in the amount of less phosphorylated NF-H may induce disorganization of NF in the perikarya and probably also in the axons, possibly leading to axonal degeneration, muscle atrophy and finally death provoked by the impairment of axonal transport (Meiri *et al.*, 1993; Gotow, 2000). Furthermore, with the observation of a strong degree of immunostaining for phosphorylated NF epitopes in both untreated and aluminium-injected animals, it was suggested that protein subunits that are already present in the neurons under normal circumstances are recruited, in the presence of aluminium, to form NF accumulation through the directed assembly of masses of oriented filaments (Forbes *et al.*, 2002).

Perikaryal NF accumulation following aluminium exposure was also found in a neuroblastoma cell line (Shea *et al.*, 1989), in PC12 cells differentiated with nerve growth factor, although to a less extent (Shea and Fischer, 1991), or in cultured rat dorsal root ganglia explants (Gilbert *et al.*, 1992). NF accumulation was also observed in abnormal neurites following intracerebral administration of aluminium to the rat brain (Shigematsu and McGeer, 1992). Following chronic aluminium treatment aggregation of cytoskeletal proteins was found in various regions of the rat brain (Kaur *et al.*, 2006). Moreover, it was reported that aluminium can cause aggregation of purified bovine, rabbit and rat NF *in vitro* (Troncoso *et al.*, 1990). It was suggested that free aluminium ions, acting as bridges among charged residues (namely phosphate groups) on neighbouring filaments, may cause cross-linking and accumulation of NF in neuronal perikarya (Gilbert *et al.*, 1992). In fact, it was reported that aluminium binding to phosphorylated NF-M induces conformational changes that resulted in conformations containing high β–pleated-sheet contents, which precipitate on standing (Hollosi *et al.*, 1992; Shen *et al.*, 1994). Additionally, according to *in vitro* experiments presented by Leterrier *et al.* (1992) aluminium alone, or in combination with maltol or other chelators, increases the phosphorylation of NF-M and NF-H subunits and induces the aggregation of NF. These authors, using purified NF

incubated with aluminium, saw that the binding of aluminium to the arm-like projections of the NF-H and NF-M subunits caused a conformational change of the molecule (intrafilamentous reaction), and that it strongly stimulated the interaction between NF (interfilamentous reaction) (Leterrier *et al.*, 1992).

Indeed, aluminium can induce neurofilamentous aggregates by a number of mechanisms, from the level of gene expression (altered gene expression) to terminal NF proteolysis. The expression of mRNA coding for NF subunits is reduced in aluminium intoxicated animals (Muma *et al.*, 1988) and also in aluminium treated cultured neuronal cells (Gilbert *et al.*, 1992). NF-H mRNA levels were found to be decreased in neurons containing aluminium-induced NF accumulation (Chambers and Muma, 1997). The authors suggested that the NF-H gene expression may be down regulated by an inhibitory feedback mechanism induced by perikaryal accumulation of NF. Moreover, Nixon and Sihag (1991) proposed that premature phosphorylation of C-terminal tails of NF in the cell body might prevent their entry into the axon. Impairment of axonal transport of NF was also observed in rabbits following aluminium treatment (Troncoso *et al.*, 1985), which may reflect abnormalities of NF phosphorylation and NF interactions. Aluminium-induced aggregation of NF-H and NF-M subunits is dependent upon their phosphorylation state (Shea *et al.*, 1992b). Increased addition of phosphate groups to NF-H proteins was observed *in vivo* following the administration of aluminium in drinking water to rats (Johnson and Jope, 1988). The aberrant state of phosphorylation appears to be due to the altered activity of cdk5/p35 kinase (Lew *et al.*, 1994), a member of the cyclin-dependent kinase-5 (cdk5) family which appears to be predominantly responsible for the phosphorylation of KSP motifs in NF tail (Hisanaga *et al.* 1991; Shetty *et al.*, 1993; Veerana *et al.*, 1995). Furthermore, the activity of protein kinase C, a kinase among others involved in the phosphorylation of NF head, was also found to increase due to aluminium in rat brain (Johnson *et al.*, 1990). On the other hand, aluminium also contributes to abnormal NF phosphorylation by inhibiting endogenous phosphatase activity (Shetty *et al.*, 1992). In addition, it was observed that following intracisternal $AlCl_3$ inoculation, a dose-dependent inhibition of NF-H dephosphorylation by exogenous phosphatases was induced (Strong and Jakowec, 1994). Spinal motor neuron neuroaxonal spheroids after chronic aluminium neurotoxicity were found to contain phosphatase-resistant NF-H (Gaytan-Garcia *et al.*, 1996). Moreover, following chronic aluminium treatment the levels of

phosphoprotein phosphatases were found to be depleted in the cerebral cortex (Kaur *et al.*, 2006). Effects on NF proteolysis were also reported. It was demonstrated that aluminium caused inhibition of proteolysis of NF in cytoskeleton preparations (Nixon *et al.*, 1990). Shea and co-workers (1992a) showed that *in vitro* aluminium induced the resistance of NF to calpain-mediated proteolysis. Also, NF isolated from intact cells treated with aluminium were found to be resistant to *in vitro* degradation by endogenous calcium-dependent proteases (Shea *et al.*, 1995). Therefore, an increase in the amount of NF may occur even when NF proteins are less expressed. In concert with the phosphorylation described earlier, aluminium will thus potentially induce a pool of highly or aberrantly phosphorylated NF resistant to dephosphorylation and proteolysis (Strong *et al.*, 1996).

I.4. AIMS

Aluminium is the most widely distributed metal in the environment and is extensively used in modern life. Aluminium enters the human body from the environment and from diet and medication. However, there is no known physiological role for aluminium within the body and hence this metal may produce adverse physiological effects. Aluminium has been related to neurodegenerative diseases, including dialysis encephalopathy, ALS/PD and AD. The impact of aluminium on the nervous systems is broad, including abnormalities in cytoskeletal proteins, behaviour, neurotransmission systems, energy metabolism, second messengers, and is also known to induce oxidative damage and neuronal apoptosis. Thus, the study of aluminium neurotoxicity is of major importance. Altered protein phosphorylation is also correlated with neurodegenerative disorders. Indeed, proteins such as neurofilaments (NF) or tau are found abnormally phosphorylated in AD brains. This alteration of protein phosphorylation might be due to changes in protein phosphatase expression or activity. Moreover, it has been reported that aluminium contributes to the abnormal phosphorylation of these proteins. Therefore, the study of the relation between aluminium toxicity and alteration in protein phosphatase 1, and consequently on the cellular signal transduction pathways involved, was the general aim of this dissertation.

The first approach was to evaluate the effect of aluminium on cellular viability and on PP1 expression and activity. Thus, a comparative study was performed using two cell lines with non-neuronal or neuronal-like characteristics. This work is presented in Chapter II. Given that aluminium is known to induce neurotoxicity, the next step was to evaluate the aluminium effect in a neuronal system. For that, cortical neuronal primary cultures were used. In this system, the alterations observed under aluminium exposure on cellular viability were monitored, as well as changes on the expression of the proteins PP1, neurofilaments and synaptophysin. This study is described in Chapter III. The discovery of other proteins whose expression may be altered due to aluminium was another goal that was achieved using SELDI-TOF MS technology. This data, obtained using *in vitro* and *in vivo* systems is presented in Chapter IV.

CHAPTER II

EFFECT OF ALUMINIUM ON PP1 EXPRESSION AND ACTIVITY IN PC12 AND COS-1 CELL LINES

II. EFFECT OF ALUMINIUM ON PP1 EXPRESSION AND ACTIVITY IN PC12 AND COS-1 CELL LINES

II.1. INTRODUCTION

Several studies have implicated aluminium with neurodegenerative diseases such as ALS/PD and AD. The finding of high aluminium contents in AD brain, especially in the core of senile plaques and associated with neurofibrillary tangles have been responsible for the aluminium hypothesis in this disease. Moreover, the neurotoxicity observed in animals exposed to aluminium has been shown to mimic many of the pathophysiological features of AD. Abnormal phosphorylation of proteins has also been related with neurodegenerative diseases and associated processes. This anomaly is a consequence of altered protein kinase and/or protein phosphatase (PP) expression or activity. The contribution of aluminium to these processes has also been reported.

In this chapter we aim to evaluate aluminium involvement in the inhibition of protein phosphatases and neurodegeneration. For that purpose the effect of aluminium was evaluated on cellular viability, on the expression of two PP1 isoforms, and on PP1 activity. Two cell lines were used in a comparative study: PC12 with neuronal-like properties and COS-1 a non-neuronal cell line.

It is known that aluminium binds strongly to oxygen-donor ligands such as phosphate, and to serum proteins such as transferrin. Thus, all the experiments with aluminium were performed in phosphate-free medium. Also, because the composition of serum (FBS or HS) is not fully defined and some aluminium ligands may be present, all the aluminium experiments were also performed with serum-free medium. The experiments with serum were performed only as a control, for comparative purposes.

II.2. MATERIALS AND METHODS

The complete composition of all solutions and media used, as well as other relevant information, is presented in Appendix I. All reagents were of cell culture grade or ultra pure. Detailed methods are also described in Appendix II.

II.2.1. Cell culture

In this study, two cell lines were used, a rat adrenal pheochromocytoma cell line (PC12), with neuronal-like properties, and a non-neuronal monkey kidney cell line (COS-1). PC12 cells were cultured in complete RPMI 1640 medium: RPMI 1640 (Gibco, Invitrogen) supplemented with 10% (v/v) heat-inactivated horse serum (HS) (Gibco, Invitrogen), 5% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, Invitrogen), 1% (v/v) antimycotic-antibiotic solution (10000 U/ml penicillin, 10000 µg/ml streptomycin, 25 µg/ml amphotericin B in 0.85% saline) (Gibco, Invitrogen) and 0.85 g/L $NaHCO_3$. COS-1 cells were maintained in complete DMEM (Dulbecco's modified Eagle's medium): DMEM (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) antibiotic-antimycotic solution and 3.7 g/L $NaHCO_3$. Both cell lines were grown routinely in 100 mm cell culture plates and maintained at 37 ºC in an atmosphere of 5% CO_2. For passaging, COS-1 cells were dissociated with trypsin-EDTA (Gibco, Invitrogen). For experimental procedures, cells were collected, counted and seeded onto 6-well plates. When using PC12 cells, the plates were previously treated with 100 µg/ml poly-L-ornithine (Sigma-Aldrich) for 10 min and washed 3 times with sterile deionised water.

II.2.2. Experimental cell treatments

II.2.2.1. Exposure to aluminium

To evaluate the aluminium effects on PP1α and PP1γ_1 expression in PC12 and COS-1 cells, both cell lines were plated onto 6-well plates and were allowed to reach 80%

confluency. Cells were then washed twice with serum-free and phosphate-free medium, RPMI (Biosource) or DMEM (Sigma-Aldrich), depending on the cell line. Cells were subsequently incubated with, respectively: phosphate-free medium with serum (10% HS and 5% FBS or 10% FBS), serum-free and phosphate-free medium or aluminium solution (10 mM $AlCl_3.6H_2O$/50 mM HEPES pH 7.4 in phosphate-free medium) at final concentrations of 0.1 mM, 0.5 mM or 1 mM. Cells were incubated at 37 ºC for varying times (12, 24, 36 or 48 h). A control sample, without treatment, collected at time 0 h was also included. Samples were collected and immunoblotting was performed as described below. Another set of cells treated as above was used to evaluate cellular viability using the MTT assay.

II.2.2.2. Recovery study

To evaluate if the effects of aluminium on PP1 expression were reversible, a recovery experiment was also performed using both cell lines. PC12 and COS-1 cells were incubated with aluminium at final concentrations of 0.5 and 1 mM for 24 h in serum-free and phosphate-free RPMI or serum-free and phosphate-free DMEM, respectively. After incubation the medium with aluminium was removed from two sets (from a total of three sets) and replaced with fresh phosphate-free and serum-free medium (without serum) in one set and with fresh complete medium (with serum) in another set. To the third set (the one which medium was not removed), fresh serum (10% HS and 5% FBS for PC12 or 10% FBS for COS-1) was added to the conditioned medium (with aluminium). All three sets were further incubated for another 24 h. A control of cells incubated with serum-free and phosphate-free medium (without treatment) for 48 h was also included. Samples were collected and immunoblotting was performed as described below. Cellular viability was also evaluated with the MTT assay using control sets of cells treated as above.

II.2.3. Cellular viability

Cellular viability was evaluated by the MTT assay. This method is based on the reduction of MTT, a water soluble tetrazolium salt, by mitochondrial dehydrogenase, to an

insoluble intracellular purple formazan. The extent of reduction of MTT was measured spectrophotometrically at 570 nm, according to Mossman (1983).

After cell treatment, the conditioned medium was removed and 0.5 mg/ml MTT (Sigma-Aldrich) solution (in serum-free RPMI or serum-free DMEM) was added and incubated for 3 h at 37 ºC. The resulting insoluble formazan precipitates were solubilized with 0.04 M HCl/Isopropanol. The absorbance of the converted dye was measured at a wavelength of 570 nm in a Cary 50 spectrophotometer. The cellular viability was expressed as a percentage of O.D. values from control cells, meaning that 90% of viability denotes a 10% decrease in viability.

II.2.4. SDS-PAGE and immunoblotting

II.2.4.1. Sample collection and immunodetection

After treatment, the conditioned medium was removed; cells were washed with PBS (Phosphate Buffered Saline, Pierce) and collected in 1 ml boiling 1% SDS solution. Cell lysates were then boiled for 10 min and sonicated for 30 sec. The total protein concentration of each sample was determined using the BCA protein assay kit (Pierce), which is based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein (Smith *et al*., 1985), according to the supplier's instructions (see Appendix II). Normalized protein samples were electrophoretically separated by 12% SDS-poliacrylamide gel electrophoresis (SDS-PAGE) using a discontinuous buffer system (Laemmli, 1970). Molecular weight markers (Kaleidoscope Prestained or Prestained SDS-PAGE Standards-Broad Range, BioRad) were used. Separated proteins were electrotransferred onto nitrocellulose membranes (Burnette, 1981) followed by immunodetection of specific proteins. To reduce the background of non-specific binding sites, the immunoblots were first blocked with 5% non-fat dry milk in 1x TBS-T (Johnson *et al*., 1984) followed by the incubation with primary antibody for 2 h at room temperature (RT), with antibodies CBC2C (anti-PP1α), CBC3C (anti-PP1γ) or anti-β-tubulin (used as a control). Both antibodies, CBC2C and CBC3C, were raised against specific C-terminal peptides of the PP1α and PP1γ proteins, respectively. All primary and secondary

antibodies were diluted in 3% non-fat dry milk in 1x TBS-T at the dilutions specified (Table II.1). Detection was carried out using horseradish peroxidase-linked secondary antibodies and an enhanced chemiluminescence detection system (ECL kit, Amersham Pharmacia Biotech) or using alkaline-phosphatase-linked secondary antibody and a colorimetric method (NBT/BCIP, Promega).

Table II.1 List of antibodies used, specific dilutions and detection method employed.

Target Protein	Primary Antibody	Secondary Antibody
PP1α	CBC2C dilution: 1:2500	Horseradish Peroxidase conjugated Rabbit IgG dilution: 1:5000
PP1γ_1	CBC3C dilution: 1:2500	Horseradish Peroxidase conjugated Rabbit IgG dilution: 1:5000
β-Tubulin	Anti-β-Tubulin dilution: 1 μg/ml	Alkaline phosphatase conjugated Mouse IgG dilution: 1:1000
		Horseradish Peroxidase conjugated Mouse IgG dilution: 1:5000

II.2.4.2. Quantification and data analysis

The intensity of the obtained bands (protein expression) on film, was quantified by densitometry using a GS-710 calibrated imaging densitometer and Quantity One software (BioRad). Data are expressed as means ± SEM of triplicate determinations from at least three independent experiments. Statistical significance analysis was conducted by one way analysis of variance (ANOVA) followed by the post test Student's *t* test. Unless otherwise noted, a level of statistical significance is considered * $p < 0.05$ *versus* control.

II.2.5. Protein phosphatase activity assays

II.2.5.1. Aluminium IC_{50} for PP1α and $PP1\gamma_1$

The effect of aluminium on purified PP1 isoform activity was assessed using ^{32}P-phosphorylase *a* as substrate. The substrate was prepared from phosphorylase *b* (Sigma) using [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech) and phosphorylase kinase (Sigma) as previously described (Watanabe *et al.*, 2003). Aluminium stock solution was diluted in 50 mM Tris-HCl, pH 7.5 containing 0.1 mM EGTA, 0.03% (v/v) Brij-35 and 0.1% (v/v) 2-mercaptoethanol (buffer B) to the final concentrations just before use. PP1 and PP2A catalytic subunits diluted in 50 mM Tris-HCl, pH 7.5 containing 1 mM $MnCl_2$, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol and 1 mg/ml BSA (buffer A) were incubated with aluminium for 5 min at 30 ºC. The reaction was started with the addition of ^{32}P-phosphorylase *a* (3 mg/ml) to a final volume of 30 µl. After 10 min, 100 µl of ice-cold 20% (w/v) TCA were added and the mixture centrifuged at 12000 x *g* for 2 min at RT. The ^{32}P-phosphate released into the supernatant was measured in a scintillation counter. The control phosphatase activity was 10-20% of total substrate radioactivity to ensure linearity. The appropriate range of concentrations was used to calculate the IC_{50} using the BioDataFit 1.02 software.

II.2.5.2. PC12 and COS-1 incubation with aluminium

To evaluate the effect of aluminium on PP activity, PC12 and COS-1 cells were plated onto 6-well plates in complete RPMI or DMEM and allowed to reach 80% confluence. The medium was removed and cells were washed 2 times with serum-free and phosphate-free medium followed by incubation with aluminium solution in serum-free and phosphate-free RPMI or DMEM at final concentrations of 0.5 or 1 mM for 24 h. After the incubation, cells were washed with cold 50 mM Tris/0.1 mM EDTA solution, scraped and collected in 1 ml Tris/EDTA solution. The cellular suspension was centrifuged at 310 x *g* for 5 min at 4 ºC and the pellet was ressuspended in 1 ml of cold homogenization buffer (see Appendix I). The total protein concentration from each sample was determined by the BCA protein assay.

II.2.5.3. Phosphatase activity assays cell extracts

The total phosphatase activity of the control and aluminium incubated cell extracts was determined by assaying the appropriate dilution (1/5 for PC12 and 1/3 for COS-1) in 50 mM Tris-HCl, pH 7.5 containing 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol and 1 mg/ml BSA, with the ^{32}P-phosphorylase *a*. The PP2A activity was determined by pre-incubating the cell extracts with 200 nM I-2 (New England Biolabs) for 15 min at 30 ºC before adding the substrate. The PP1 activity in the cell extracts was calculated as the total phosphatase activity minus the PP2A activity towards the ^{32}P-phosphorylase *a* substrate.

II.3. RESULTS

II.3.1. Cellular viability

Cellular viability was evaluated with the MTT assay (Mossman, 1983) in PC12 and COS-1 cells. The effect of serum withdrawal and the effect of increasing aluminium concentrations for different periods of incubation were evaluated, as well as the recovery after removal of aluminium.

II.3.1.1. Effect of serum withdrawal on cellular viability

The cellular viability and the effect of serum withdrawal were evaluated with time in both cell lines. Data is presented as % of control (the levels of cellular viability obtained at time 0 h, without treatment, were taken as 100%), a relation between MTT reduction and total protein concentration was performed to adjust the changes of viability induced by cell proliferation. Statistical differences are presented in relation to the control.

Although, slightly significant differences were observed, the cellular viability in PC12 cells incubated with complete medium (medium with serum) remained almost unaltered during time, exhibiting values of 87% ± 2.6%, 93% ± 4%, 90% ± 2.3% and 84% ± 1.9% after 12 h, 24 h, 36 h and 48 h of incubation, respectively (Fig. II.1). Incubation with medium without serum induced a similar response, revealing also slightly significant

decreases of the cellular viability to 81% ± 2.8% after 12 h, to 89% ± 4% after 24 h, to 91% ± 4.1% after 36 h, and to 91% ± 3.9% after 48 h of incubation. In spite of the significant changes observed in relation to the control, for both conditions (presence or absence of serum in the medium), serum withdrawal (when comparing both conditions) did not induced significant variations on cellular viability, for all time points studied in PC12 cells.

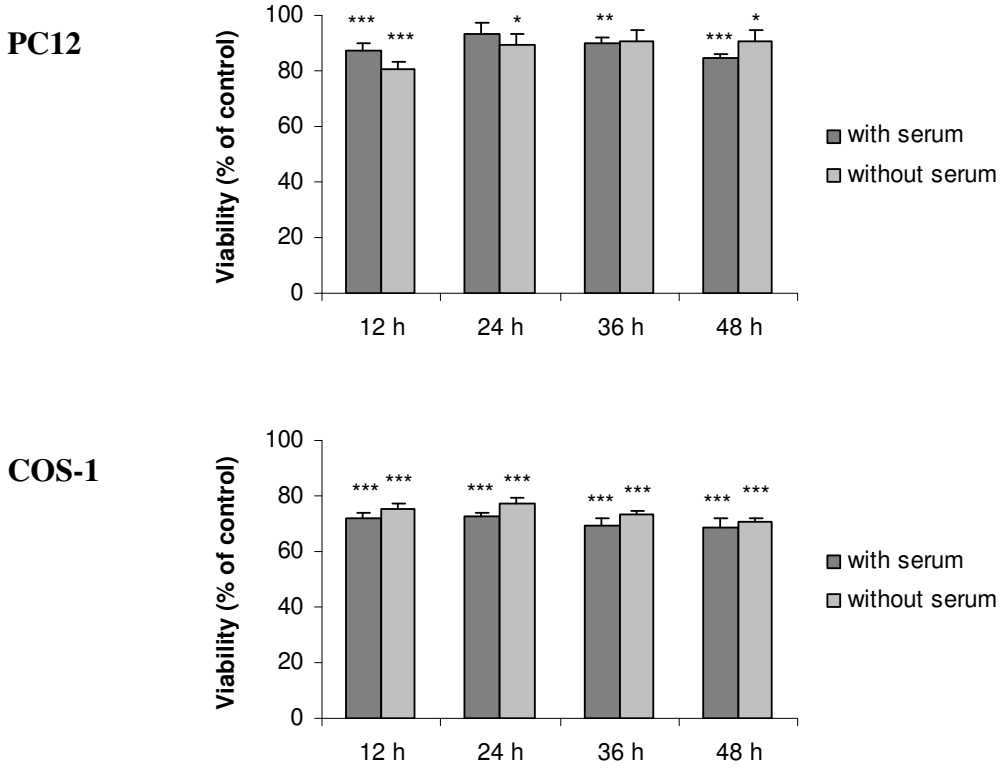

Fig. II.1 – Effect of serum withdrawal on cellular viability of PC12 and COS-1 cells as a function of time. PC12 and COS-1 cells were incubated with or without serum in the respective medium. Results are expressed as percentage of control (time 0 h is 100%) as means ± SEM of triplicate determinations from at least three independent experiments. *, **, *** Value statistically different from the control ($p < 0.05$, $p < 0.01$, $p < 0.001$, respectively).

The cellular viability in COS-1 cells incubated with complete medium (medium with serum) significantly decreased for all time points by about 30%. The cellular viability remained constant over time and the values were of 72% ± 1.8%, 73% ± 0.9%, 70% ± 2.3% and 69% ± 3.3% after 12 h, 24 h, 36 h and 48 h, respectively (Fig. II.1). A similar

response on cellular viability was observed in the same cells incubated in medium without serum, over time, showing a significant decrease of around 25% and keeping constant afterwards (75% ± 1.7%, 77% ± 2%, 73% ± 1.2% and 71% ± 1.5% after 12h, 24 h, 36 h and 48 h of incubation, respectively). Even though a decrease was observed for both conditions when related to the control, the serum withdrawal did not induce any change on the cellular viability, as verified for PC12 cells.

II.3.1.2. Effect of aluminium on cellular viability with time of exposure

The effect of $AlCl_3$ at increasing concentrations (0.1 mM, 0.5 mM or 1 mM) was also evaluated during time (24 h and 48 h), in both cell lines. Data is shown as % of control (the levels of cellular viability obtained for cells incubated without aluminium, at each time, were taken as 100%). Statistical differences are presented in relation to the control.

Aluminium induced significant decreases of cellular viability after 24 h and 48 h exposure in PC12 cells. No significant variations were observed for different aluminium concentrations in both periods (Fig. II.2). A decrease of cellular viability was observed at 24 h for all aluminium concentrations (to 79% ± 7.4% for 0.1 mM, 75% ± 2.8% for 0.5 mM and 73% ± 3.7% for 1 mM). After 48 h, cellular viability decreased to 57% ± 8.1% for 0.1 mM, to 44% ± 3.9% for 0.5 mM and to 41% ± 4.2% for 1 mM $AlCl_3$.

In COS-1 cells a similar response to aluminium treatment was observed for both periods of incubation with the same aluminium concentrations (Fig. II.2). No significant effect was observed with the exposure to 0.1 mM $AlCl_3$ for 24 h or 48 h, whereas statistically significant decreases of cellular viability were observed with 0.5 mM $AlCl_3$ and 1 mM $AlCl_3$, in both periods. Cellular viability decreased to 83% ± 2% for 0.5 mM and to 71% ± 1.3% for 1 mM after 24 h and to 83% ± 3.4% for 0.5 mM and to 64% ± 3.6% for 1 mM $AlCl_3$ after 48 h of incubation.

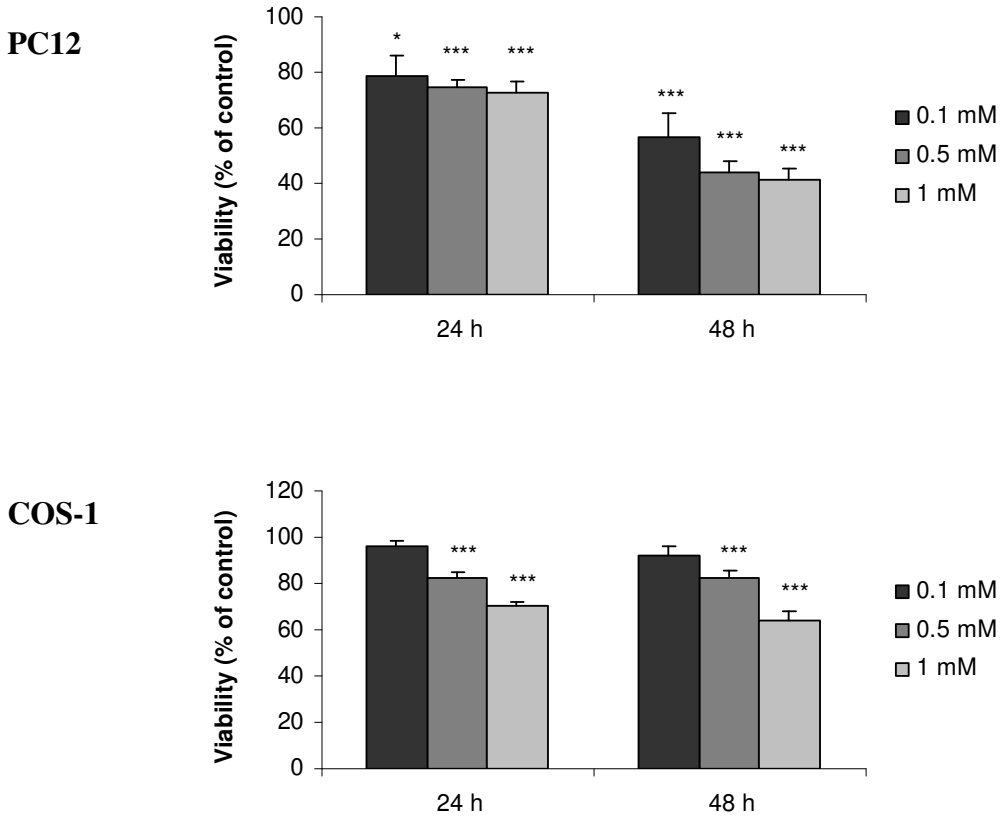

Fig. II.2 – Aluminium effect on cellular viability in PC12 and COS-1 cells. PC12 and COS-1 cells were incubated with increasing aluminium concentrations for different times. Results are expressed as percentage of control (absence of aluminium at each time) as means ± SEM of triplicate determinations from at least three independent experiments. *, ***, Value statistically different from the control ($p < 0.05$, $p < 0.001$, respectively).

II.3.1.3. Effect of aluminium withdrawal on cellular viability

Both cell lines were allowed to recover from 24 h incubation with 0.5 mM and 1 mM $AlCl_3$ by replacement of experimental medium with medium without serum, medium with serum or by the addition of 5% FBS plus 10% HS for PC12 cells, or 10% FBS for COS-1 cells, directly to the experimental medium and allowing them to recover for another 24 h. Data is presented as % of control, i. e. 100% was defined as the level of cellular viability obtained for cells incubated without aluminium for 48 h. Statistical differences are expressed in relation to the control.

After recovery the cellular viability reached values similar to control in both cell lines, even rising significantly higher with the addition of FBS/HS to the PC12 cells incubated with 0.5 mM or 1 mM aluminium for 24 h, with values reaching 125% ± 6% and 124% ± 6.6%, respectively (Fig. II.3). The substitution for medium without serum in both cell lines incubated with 1 mM, also resulted in a significant increase in cellular viability, to 120% ± 6.1% for PC12 cells and 113% ± 3.4% for COS-1 cells.

Unaltered values, in relation to the control, were 125% ± 11.4% and 114% ± 7.3% for 0.5 mM "without serum" and "with serum" respectively and 120% ± 6.1% for 1 mM "with serum" for PC12 cells. For COS-1 cells the values were 111% ± 4.7%, 102% ± 2.6% and 104% ± 4.8% for 0.5 mM "without serum", "with serum" and "with FBS" respectively and 102% ± 2% and 105% ± 2.8% for 1 mM "with serum" and "with FBS".

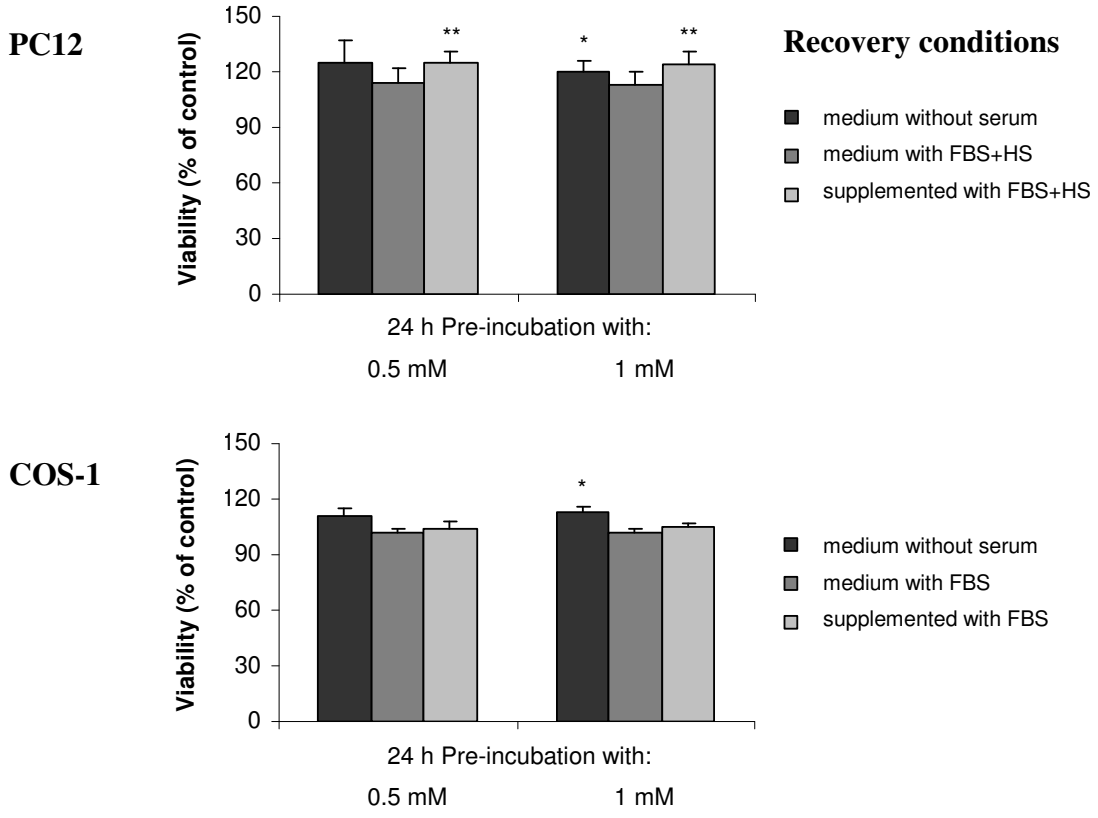

Fig. II.3 – Effect of recovery from aluminium on cellular viability in PC12 and COS-1 cells. PC12 and COS-1 cells were incubated with 0.5 mM or 1 mM aluminium for 24 h in their respective serum-free, phosphate-free medium and allowed to recover for 24 h under the indicated conditions. Results are expressed as percentage of control (absence of aluminium at 48 h) as mean ± SEM of triplicate determinations from at least three independent experiments. *, **, Value statistically different from the control ($p < 0.05$, $p < 0.01$, respectively).

II.3.2. Protein phosphatase expression

The effect of serum withdrawal and the effect of $AlCl_3$ (0.1 mM, 0.5 mM or 1 mM) on protein phosphatase (PP1α and PP1γ_1) expression was evaluated by immunoblot analysis (using specific antibodies) in both cell lines as a function of time of exposure (12 h, 24 h, 36 h and 48 h). A recovery study was also performed. The expression of β-tubulin was determined and used as a control, utilizing the same immunoblots previously used for assessing PP1 expression. No differences were detected in β-tubulin expression under the conditions tested, for both cell lines (Fig. II.4-II.9).

II.3.2.1. Effect of serum withdrawal on PP1α and PP1γ_1 expression

The expression of PP1α and PP1γ_1 and the effect of serum withdrawal were evaluated with time for both cell lines. Data is presented as % of control (the levels of expression obtained for cells collected at time 0 h, without treatment, were taken as 100%). Statistical differences are expressed in relation to the control.

In PC12 cells the expression of PP1α was not significantly altered up to 24 h in complete medium (with serum), in relation to the control. Similar results were observed when cells were incubated in medium without serum, although a slight statistically significant decrease was observed at 24 h. Comparing both conditions, serum withdrawal had no apparent effect on the expression of this protein (Fig. II.4). On the other hand, a small but reliable increase in the expression of PP1γ_1 was observed after 12 h of incubation in medium with serum, decreasing afterwards with time. The expression of PP1γ_1 in cells grown in medium without serum practically did not change with time, only a slight decrease was observed at the 24 h time point. Comparing both conditions, serum withdrawal led to an effective maintenance of PP1γ_1 expression, an effect not observed for PP1α.

The expression of PP1α in PC12 cells incubated with complete medium was 113% ± 6.4% and 100% ± 10% of control after 12 h and 24 h of incubation, and decreased significantly to 67% ± 3.8% after 36 h and to 48% ± 4.2% after 48 h of incubation. In cells incubated in serum-free medium PP1α expression was 98% ± 5.8% of control after 12 h

and a significant reduction was observed to 88% ± 5.1% after 24 h of exposure, to 70% ± 6.1% after 36 h and to 62% ± 8.6% after 48 h.

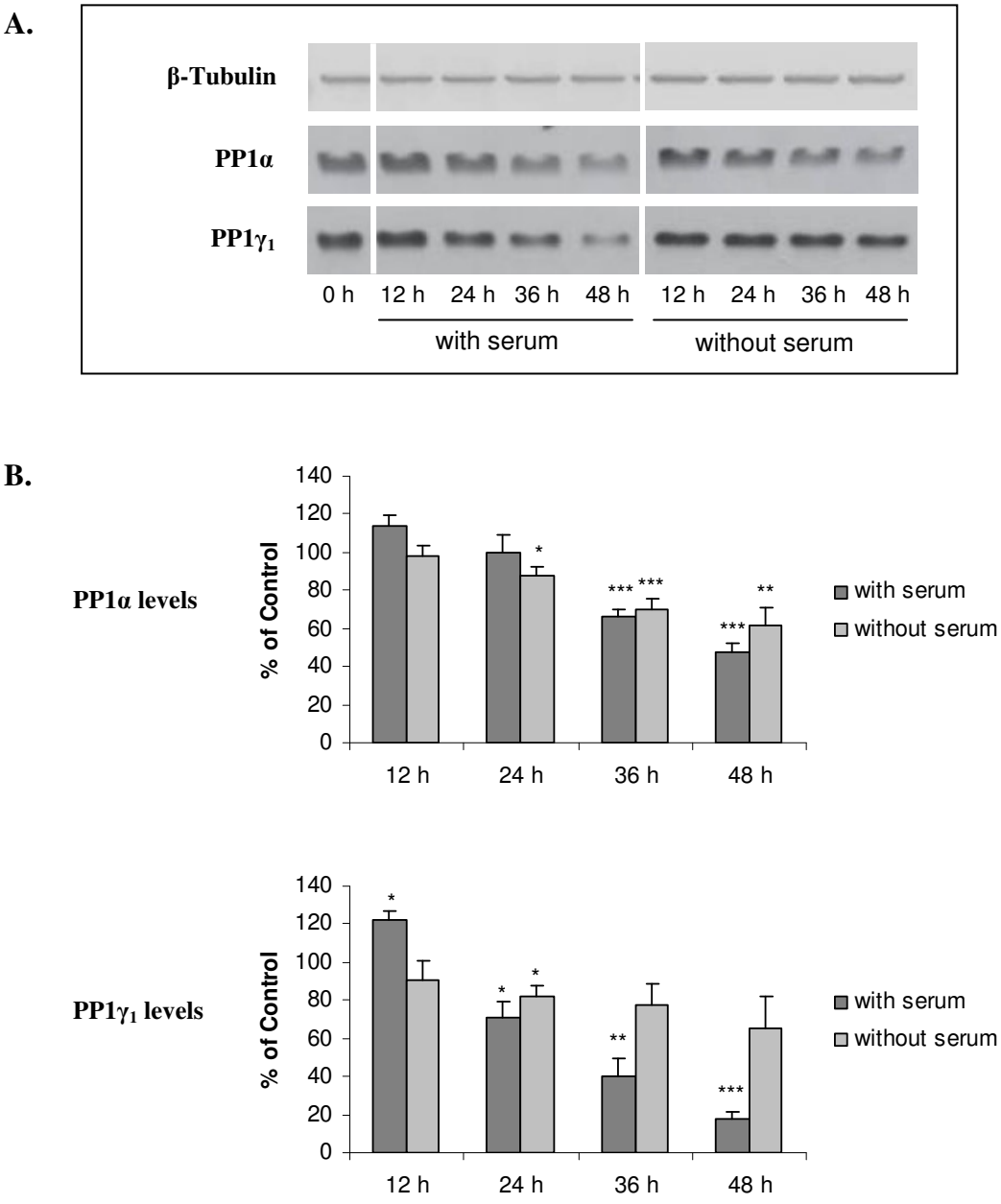

Fig. II.4 – Effect of serum withdrawal on PP1α and PP1γ_1 expression in PC12 cells. Cells were incubated with or without serum for different periods of time. The expression level of PP1α and PP1γ_1 was analysed by immunobloting with specific antibodies **(A)**. β-Tubulin levels were also assessed as a control. Results obtained from at least three independent experiments were quantified and are represented graphically **(B)** as percentage of control (time 0 h). Data are expressed as mean ± SEM (*, **, *** value statistically different from control; $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively).

PP1γ_1 expression in PC12 cells incubated in medium with serum was observed to increase to 122% ± 5.2% of control after 12 h of treatment, followed by significant decreases to 71% ± 8.7% after 24 h, to 41% ± 8.7% after 36 h and to 18% ± 4% after 48 h of incubation. Cells incubated in serum-free medium showed statistically non-significant decreases in PP1γ_1 expression to 91% ± 9.7% of control after 12 h, to 78% ± 10.9% after 36 h, to 66% ± 16.7% after 48 h, but a significant decrease after 24 h exposure to 82% ± 5.8%.

In COS-1 cells, the expression of PP1α and PP1γ_1 decreased from 24 h onwards for both media used (Fig. II.5). The expression of PP1α with time in COS-1 cells was similar to that verified in PC12 cells. COS-1 cells incubated with serum demonstrated a significant decrease in PP1α expression after 36 h (80% ± 3.9%) and 48h (67% ± 4.9%). At 12 h and 24 h the values were 125% ± 15.7% and 93% ± 7.6%, respectively. COS-1 cells incubated in medium without serum, as verified for PC12 cells, exhibited a significant decrease of PP1α expression from 24 h of incubation onwards. PP1α expression was of 103% ± 17.8% at 12 h, but decreased to 66% ± 4.8% at 24 h, to 61% ± 8.5% at 36 h and to 52% ± 7.4% at 48 h of incubation.

The expression of PP1γ_1 in COS-1 cells incubated with complete medium, remained unaltered up to 12 h (119% ± 14.4%), but significantly decreased with time to 68% ± 8.8% (24 h), to 61% ± 6.1% (36 h) and to 42% ± 5.7% (48 h). For cells incubated in medium without serum, a similar response was observed. At 12 h no statistically significant change was verified (90% ± 18.9%). Significant decreases in PP1γ_1 expression were observed after 24 h (to 38% ± 3.8%), 36 h (to 34% ± 5.5%) and 48 h of exposure (to 21% ± 3.7%).

II.3.2.2. Effect of aluminium on PP1α and PP1γ_1 expression with time

The effect of increasing concentrations of $AlCl_3$ (0.1 mM, 0.5 mM or 1 mM) on PP1α and PP1γ_1 expression were evaluated, with time (12 h, 24 h, 36 h and 48 h), in PC12 and COS-1 cell lines. Data is shown as % of control (the levels of expression obtained for cells incubated with serum free and phosphate free medium, and without aluminium, at each time, were considered as 100%). Thus, the possible effect of serum withdrawal on

PP1α and PP1γ_1 expression was suppressed, and only the effect of aluminium was evaluated. Statistical differences are expressed in relation to the control.

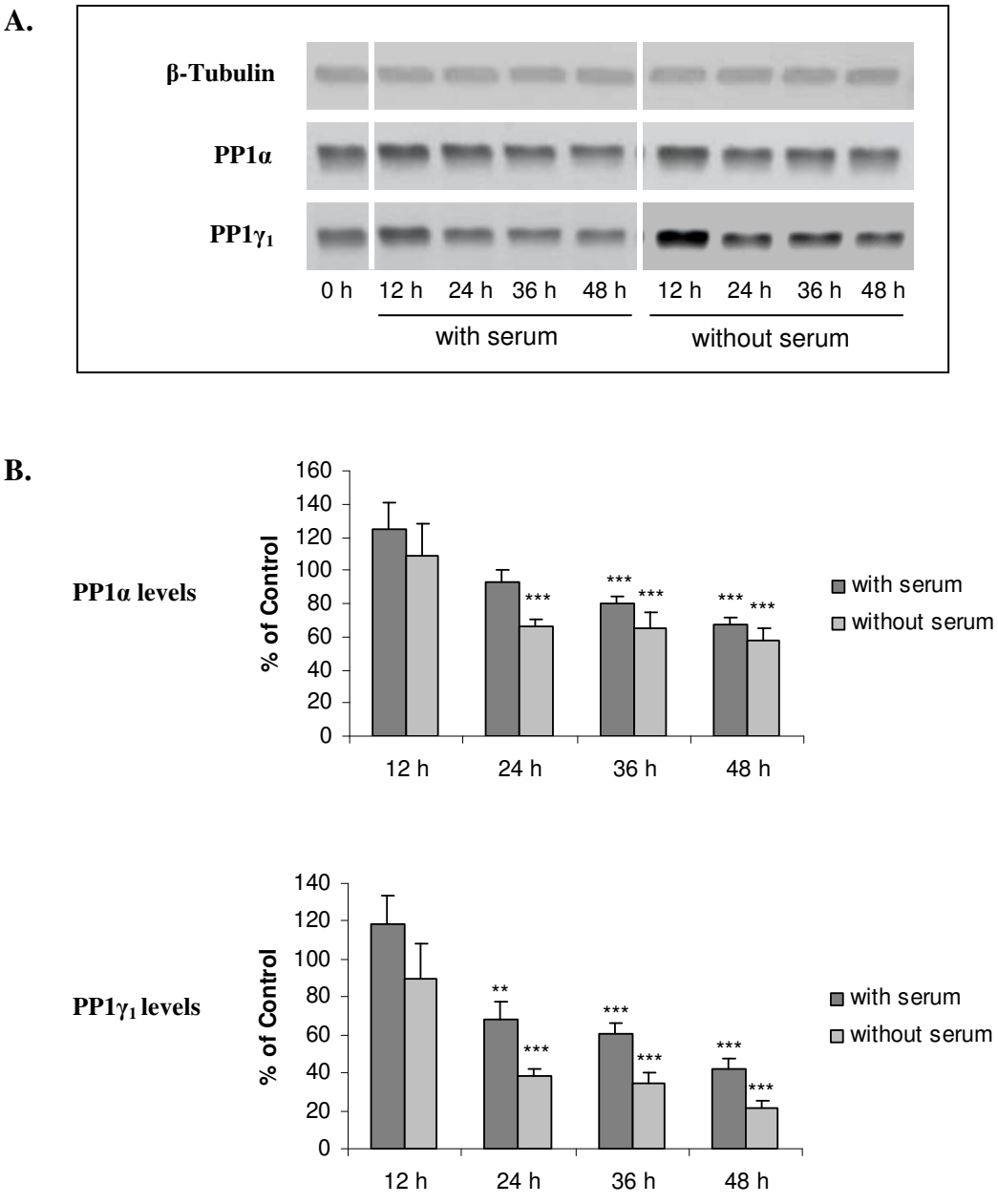

Fig. II.5 - Effect of serum withdrawal on PP1α and PP1γ_1 expression in COS-1 cells. Cells were incubated with or without serum for different periods of time. The expression levels of PP1α and PP1γ_1 were analysed by immunobloting with specific antibodies **(A)**. β-Tubulin levels were also assessed as a control. Results obtained from at least three independent experiments were quantified and are represented graphically **(B)** as percentage of control (time 0 h). Data are expressed as mean ± SEM (**, *** value statistically different from control; $p < 0.01$, $p < 0.001$, respectively).

The expression of PP1α in PC12 cells, in general, decreased with increasing aluminium concentrations and with time of exposure at each concentration (Fig. II.6).

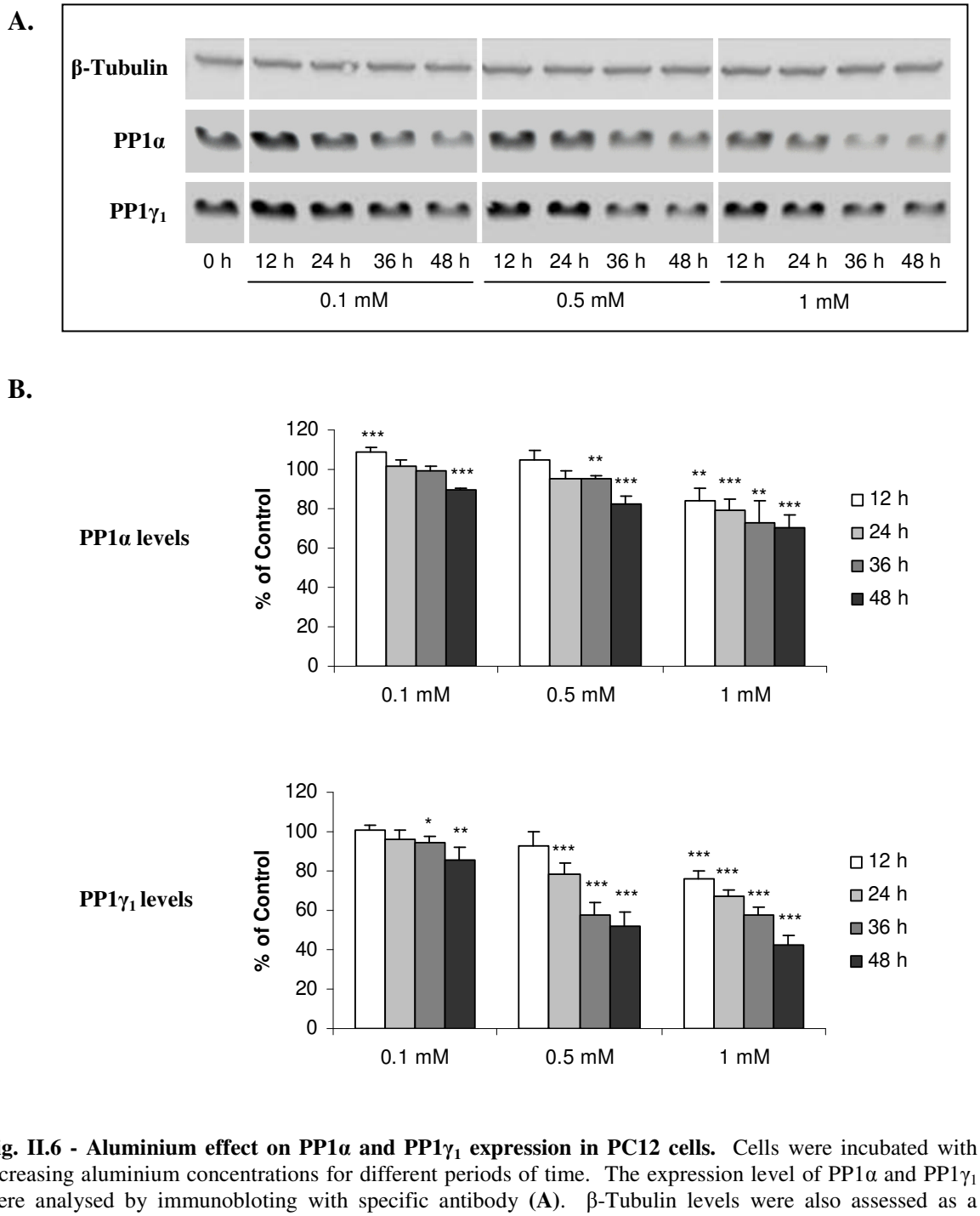

Fig. II.6 - Aluminium effect on PP1α and PP1γ_1 expression in PC12 cells. Cells were incubated with increasing aluminium concentrations for different periods of time. The expression level of PP1α and PP1γ_1 were analysed by immunobloting with specific antibody **(A)**. β-Tubulin levels were also assessed as a control. Results obtained from at least three independent experiments were quantified and are represented graphically **(B)** as percentage of control (absence of aluminium at each time). Data are expressed as means ± SEM (*, **, *** value statistically different from the control $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively).

PC12 cells incubated with 0.1 mM $AlCl_3$ presented a small but significant increase at 12 h to 109% ± 2.2% of control of PP1α expression, with a significant decrease only being observed after 48 h of exposure (90% ± 1.2%). Statistically significant decreases of PP1α expression in PC12 cells were obtained at 36 h and 48 h of treatment with 0.5 mM $AlCl_3$ (to 95% ± 1.8% and to 82% ± 4.2%, respectively). In contrast, incubation with 1 mM $AlCl_3$ produced significant decreases at all time points (to 84% ± 6% at 12 h, to 79% ± 5.4% at 24 h, to 73% ± 11.4% at 36 h and to 71% ± 6.3% at 48 h).

The incubation of PC12 cells with aluminium induced a similar effect on the pattern of expression of PP1γ_1 showing a decrease with increasing aluminium concentrations and with time, as verified for PP1α (Fig. II.6). Statistically, with 0.1 mM $AlCl_3$ PP1γ_1 expression significantly decreased only after 36 h (to 94% ± 3.7%) and 48h of treatment to (85% ± 6.8%). Whereas, incubation with 0.5 mM $AlCl_3$ yielded significant decreases earlier, from 24 h onwards (to 78% ± 5.3% at 24 h, to 58% ± 6.4% at 36 h and to 52% ± 6.8% at 48 h), 1 mM $AlCl_3$ produced significant decreases at all time points sampled (to 76% ± 4% at 12 h, to 67% ± 3.2% at 24 h, to 57% ± 4% at 36 h and to 43% ± 4.6% at 48 h).

As verified for PC12 cells, PP1α expression in COS-1 cells exhibits a similar response with increasing aluminium concentration and with time (Fig. II.7). For COS-1 cells no significant differences on the expression were observed, during time, with the incubation of 0.1 mM $AlCl_3$. Whereas incubation with 0.5 mM $AlCl_3$ yielded significant decreases evident at the earliest time point of increasing with time (to 77% ± 7.6% after 24 h, to 68% ± 4.1% after 36 h and to 53% ± 7.2% after 48 h). With 1 mM $AlCl_3$ significant decreases were observed at all time points (decrease to 77% ± 3.1% after 12 h, to 54% ± 6.4% after 24 h, to 56% ± 5.6% after 36 h and to 54% ± 4.6% after 48 h).

The effect of increasing aluminium concentration on PP1γ_1 expression in COS-1 cells with time was a reduction in PP1γ_1 expression, as verified for PC12 cells. Although for COS-1 cells, 0.1 mM $AlCl_3$ induced a small but statistically significant increase of PP1γ_1 expression at all time points (120% ± 4.2% at 12 h, 121% ± 6.8% at 24 h, 125% ± 5.7% at 36 h and 118% ± 9.7% at 48 h) (Fig. II.7). Whereas 0.5 mM and 1 mM $AlCl_3$ produced decreases in PP1γ_1 expression with time, statistically significant results were only obtained with 0.5 mM $AlCl_3$ at 48 h of exposure (decrease to 55% ± 17.3%), but at all

time points for 1 mM $AlCl_3$ (decrease to 72% ± 7.8% at 12 h, to 65% ± 5% at 24 h, to 59% ± 8.1% at 36 h and to 41% ± 18.3% at 48 h).

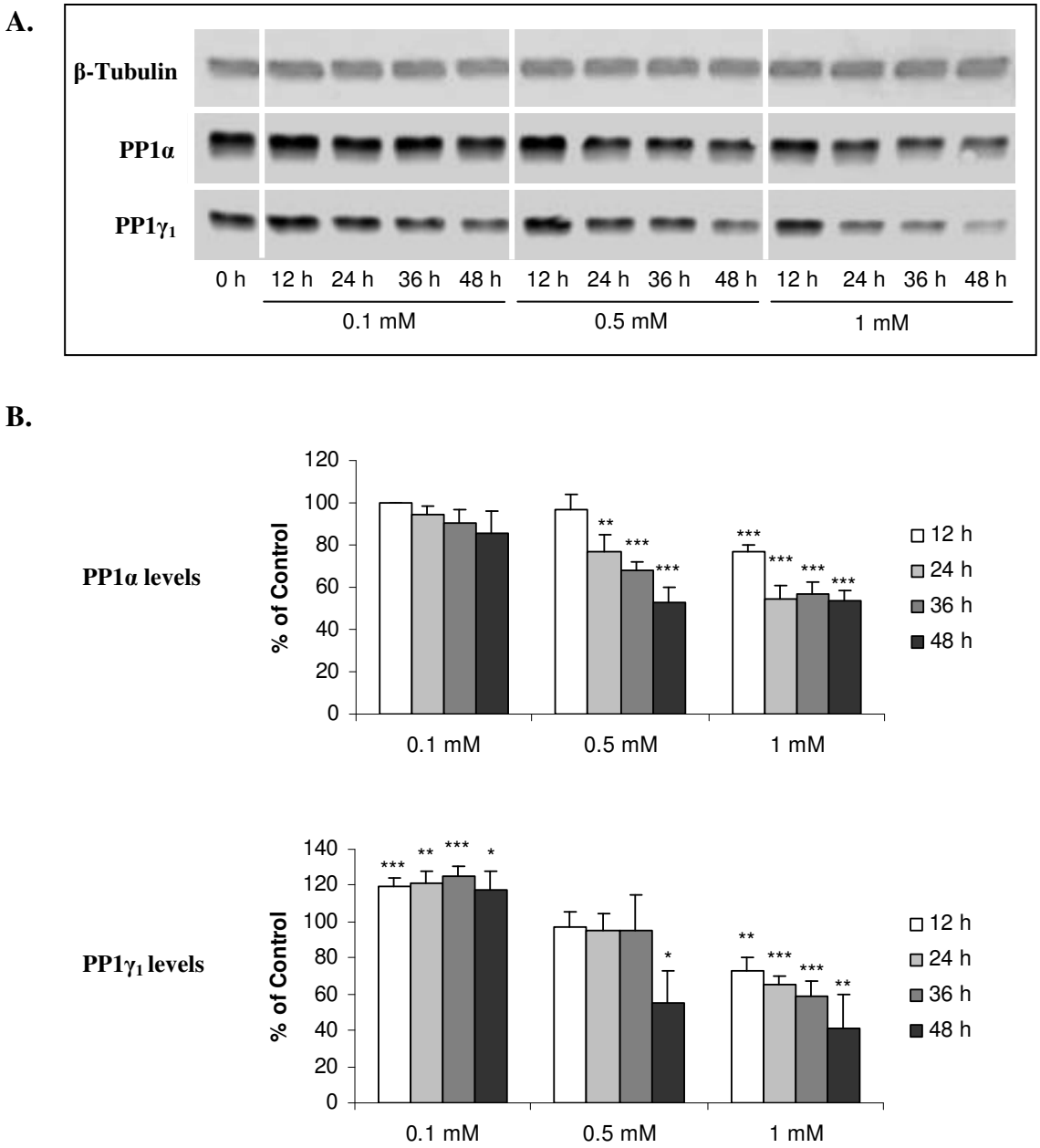

Fig. II.7 - Effect of aluminium on PP1α and PP1γ_1 expression in COS-1 cells. Cells were incubated with increasing aluminium concentrations for different periods of time. The expression levels of PP1α and PP1γ_1 were monitored by immunobloting with specific antibodies **(A)**. β-Tubulin levels were also assessed as a control. Results obtained from at least three independent experiments were quantified and are represented graphically **(B)** as percentage of control (absence of aluminium at each time). Data are expressed as mean ± SEM (*, **, *** value statistically different from control; $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively).

II.3.2.3. Effect of aluminium withdrawal on PP1α and PP1γ_1 expression

A recovery study was also performed in relation to the expression of protein phosphatases, similar to that performed for cellular viability. After 24 h incubation with 0.5 mM and 1 mM $AlCl_3$, both cell lines were allowed to recover by replacement of the experimental medium with medium without serum, with medium with serum or by the addition of 5% FBS plus 10% HS (for PC12 cells, but just 10% FBS for COS-1 cells) directly to the experimental medium. Recovery was allowed for an additional 24 h of incubation. Data is presented as % of control, i. e. 100% was the level of expression of protein phosphatases obtained for cells incubated without aluminium for 48 h. Statistical differences are expressed in relation to the control.

In PC12 cells the expression of PP1α following recovery was generally higher than the control (Fig.II.8), revealing a statistically significant increase for all conditions tested, except for the substitution for medium without serum in cells incubated with 1 mM $AlCl_3$ that yielded an expression similar to the control. The levels of PP1α expression were 112% ± 4.4%, 129% ± 6.6% and 122% ± 6.8% for cells incubated with 0.5 mM $AlCl_3$ for 24 h and allowed to recover for another 24 h in medium without serum, in medium with serum or with the addition of FBS/HS to the medium, respectively. For the cells incubated with 1 mM $AlCl_3$ and allowed to recover in similar conditions, the levels of expression achieved were 106% ± 7.1%, 120% ± 7.1% and 116% ± 6.5%, respectively (Fig.II.8). The highest level of PP1α expression was induced by medium with serum in cells previously incubated with 0.5 mM $AlCl_3$ for 24 h.

On the other hand, for both aluminium concentrations, the substitution to medium without serum induced PP1γ_1 expression levels similar to the control (93% ± 3.8% for 0.5 mM $AlCl_3$ and 93% ± 4.7% for 1 mM $AlCl_3$) (Fig.II.8). However, the substitution to medium with serum and the addition of FBS/HS to the medium yielded significant decreases in the expression levels of PP1γ_1 for both aluminium concentrations (77% ± 4.8% and 78% ± 5.4% respectively for 0.5 mM $AlCl_3$, and 69% ± 5.5% and 66% ± 5.4% respectively for 1 mM $AlCl_3$) (Fig.II.8).

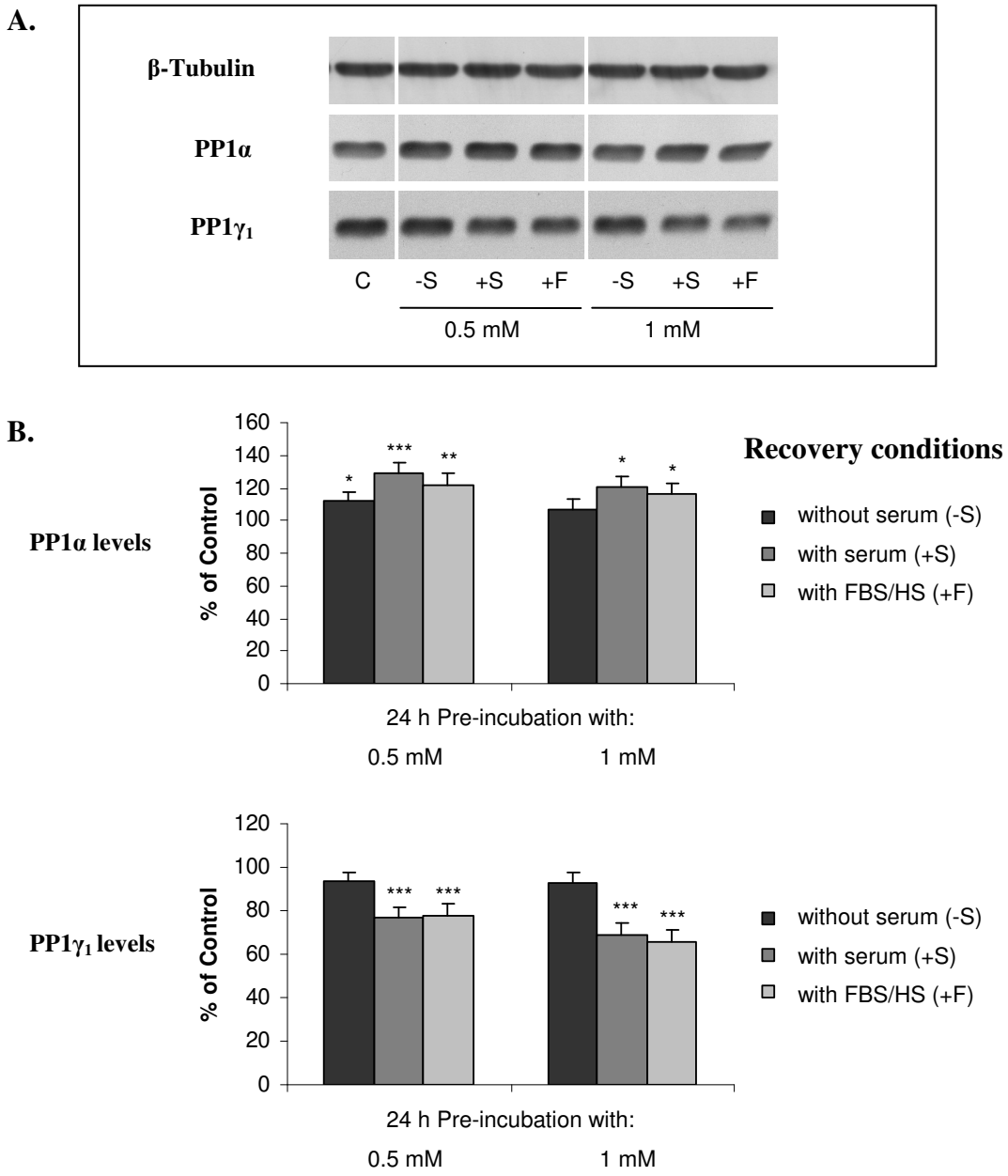

Fig. II.8 – Effect of aluminium withdrawal on PP1α and PP1γ_1 expression in PC12 cells. Cells were incubated with 0.5 mM or 1 mM aluminium for 24 h in serum-free and phosphate-free medium, and allowed to recover for another 24 h by: substituting experimental medium with fresh medium without serum and phosphate **(-S)**, or fresh complete medium with serum **(+S)**, or by adding fresh 5% FBS and 10% HS to the experimental medium **(+F)**. The expression levels of PP1α and PP1γ_1 were analysed by immunobloting with specific antibodies **(A)**. β-Tubulin levels were also assessed as a control. Results obtained from at least three independent experiments were quantified and are represented graphically **(B)** as percentage of control (48 h without treatment) and expressed as mean ± SEM (*, **, *** value statistically different from control; $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively).

In COS-1 cells exposed to either aluminium concentration, the substitution to medium with serum induced the highest levels of recovery, for both PP1α and PP1γ_1. In

fact, for PP1α, that was the only condition that induced a significant increase in the expression, an effect that was observed with both aluminium concentrations (124% ± 8% for 0.5 mM and 129% ± 8.7% for 1 mM) (Fig.II.9).

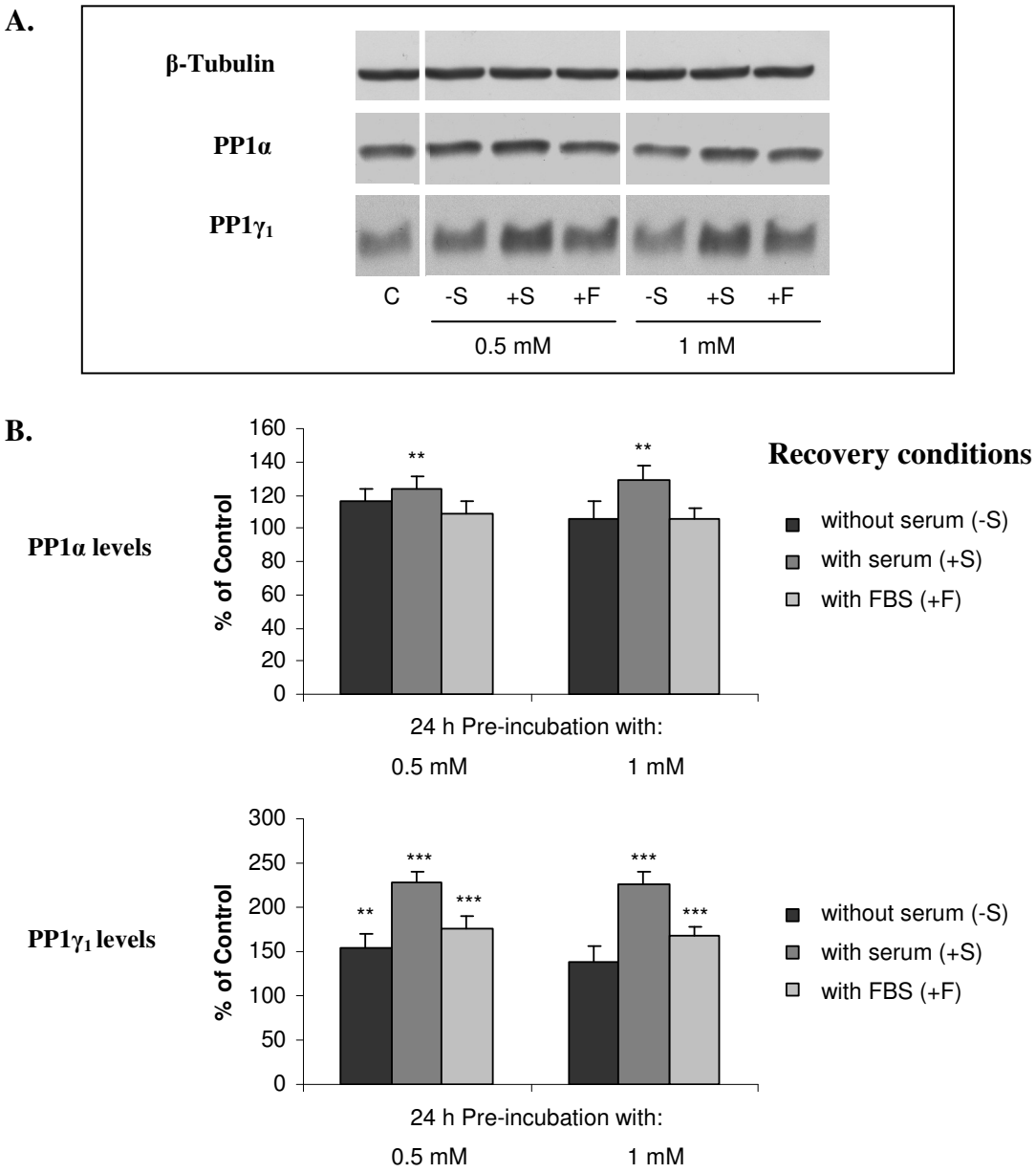

Fig. II.9 – Effect of aluminium withdrawal on PP1α and PP1γ_1 expression in COS-1 cells. Cells were incubated with 0.5 mM or 1 mM aluminium for 24 h in serum-free and phosphate-free medium, and allowed to recover for another 24 h by: substituting experimental medium with fresh medium without serum and phosphate **(-S)**, or fresh complete medium with serum **(+S)**, or by adding fresh 10% FBS to the experimental medium **(+F)**. The expression levels of PP1α and PP1γ_1 were analysed by immunobloting with specific antibodies **(A)**. β-Tubulin levels were also assessed as a control. Results obtained from at least three independent experiments were quantified and are represented graphically **(B)** as percentage of control (48 h without treatment) and expressed as mean ± SEM (**, *** value statistically different from control; $p < 0.01$, $p < 0.001$, respectively).

The other experimental recovery conditions led to expression levels similar to the control (116% ± 7.9% and 109% ± 7.4% respectively, for 0.5 mM $AlCl_3$ followed by medium without serum or addition of FBS to the experimental medium). Also, the expression levels for cells treated similarly but pre-incubated with 1 mM $AlCl_3$ were 105% ± 11.2% and 106% ± 6%, respectively. For PP1γ_1 expression, the values observed, following recovery from aluminium exposure were significantly higher than the control for all conditions tested, except for cells incubated with 1 mM whose medium was substituted by medium without serum (138% ± 18.7%). The levels of PP1γ_1 expression for cells incubated with 0.5 mM $AlCl_3$ for 24 h and whose medium was substituted by medium without serum, by medium with serum or to which FBS/HS was added, were 155% ± 15.4%, 227% ± 12.5% and 177% ± 12.9%, respectively. For cells incubated with 1 mM $AlCl_3$ the expression levels reached 226% ± 13.1% after substitution for medium with serum, and reached 169% ± 8.6% by the addition of FBS to the experimental medium (Fig. II.9).

II.3.3. Protein phosphatase activity

The effect of aluminium on PP1 activity was evaluated via different approaches. Either purified recombinant PP1α and PP1γ_1 were incubated with a range of aluminium concentrations and the IC_{50} was determined, or PC12 and COS-1 cells were incubated with 0.5 mM or 1 mM $AlCl_3$ and PP1 activity was evaluated in the cell extracts. In both approaches the ^{32}P-phosphorylase *a* was used as a substrate.

II.3.3.1. Effect of aluminium on recombinant PP1α and PP1γ_1 activity

Purified recombinant PP1α and PP1γ_1 were incubated with increasing aluminium concentrations (0.01 to 10 mM) and their activity was determined using phosphorylase *a* as a substrate. Data is presented as percentage of inhibition (Fig.II.10).

The range of aluminium concentrations used induced a decrease in both PP1α and PP1γ_1 activity, thus a characteristic aluminium inhibition curve was obtained. The IC_{50} was calculated using BioDataFit 1.02 software and approximate values were calculated for

both PP1 isoforms. The experimental values determined were: 0.277 mM for PP1α and 0.258 mM for PP1γ_1, as shown in Fig.II.10.

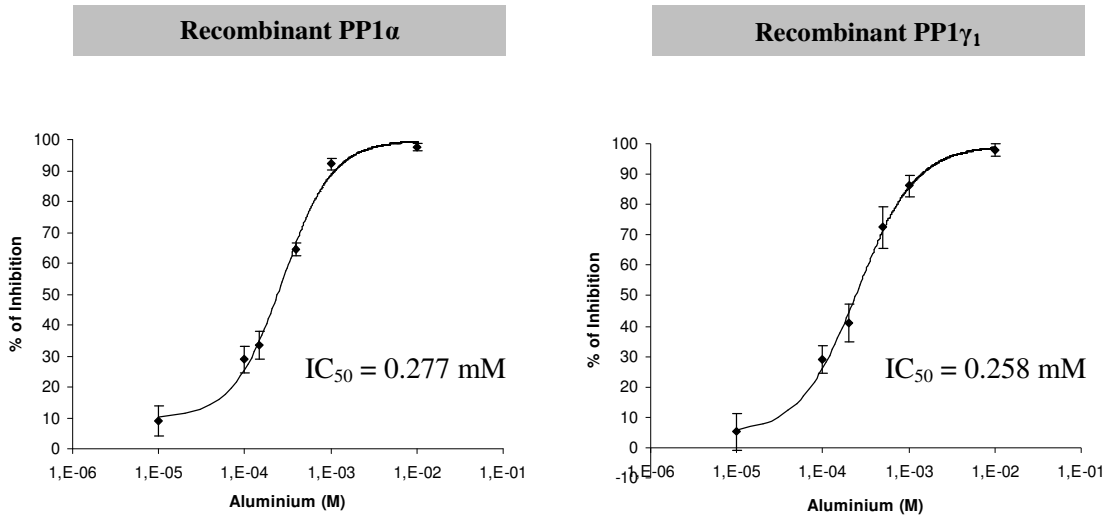

Fig. II.10 – Effect of aluminium on recombinant PP1α and PP1γ_1 activity. Purified recombinant PP1α and PP1γ_1 were incubated with increasing aluminium concentrations (0.01 to 10 mM) and their activity was determined using phosphorylase *a* as a substrate. Results obtained are presented as percentage of inhibition. Error bars are mean of duplicates of at least three independent experiments.

II.3.3.2. Effect of aluminium on cellular PP1 activity

The total protein phosphatase activity was evaluated in PC12 and COS-1 cells extracts following incubation with 0.5 mM or 1 mM $AlCl_3$ for 24 h. PP2A activity was also determined by pre-incubating the cell extracts with I-2 (a PP1-specific inhibitor). PP1 activity in the cell extracts was calculated as the total activity minus the PP2A activity towards the ^{32}P-phosphorylase *a* substrate. Data is presented as percentage of control, i. e. the level of activity obtained for cells incubated without aluminium for 24 h was considered as 100% (Fig. II.11.A and Fig. II.12.A). The partial contributions of PP2A and PP1 activities to the total activity was also calculated and data presented as percentage of total activity (Fig. II.11.B and Fig. II.12.B).

Both aluminium concentrations yielded similar decreases in the total phosphatase activity (PP total), and in PP2A and PP1 activity, in both cell lines. The decrease in PP total activity was to 75% ± 4.6% with 0.5 mM $AlCl_3$ and to 76% ± 5.6% with 1 mM $AlCl_3$ in PC12 cells (Fig. II.11).

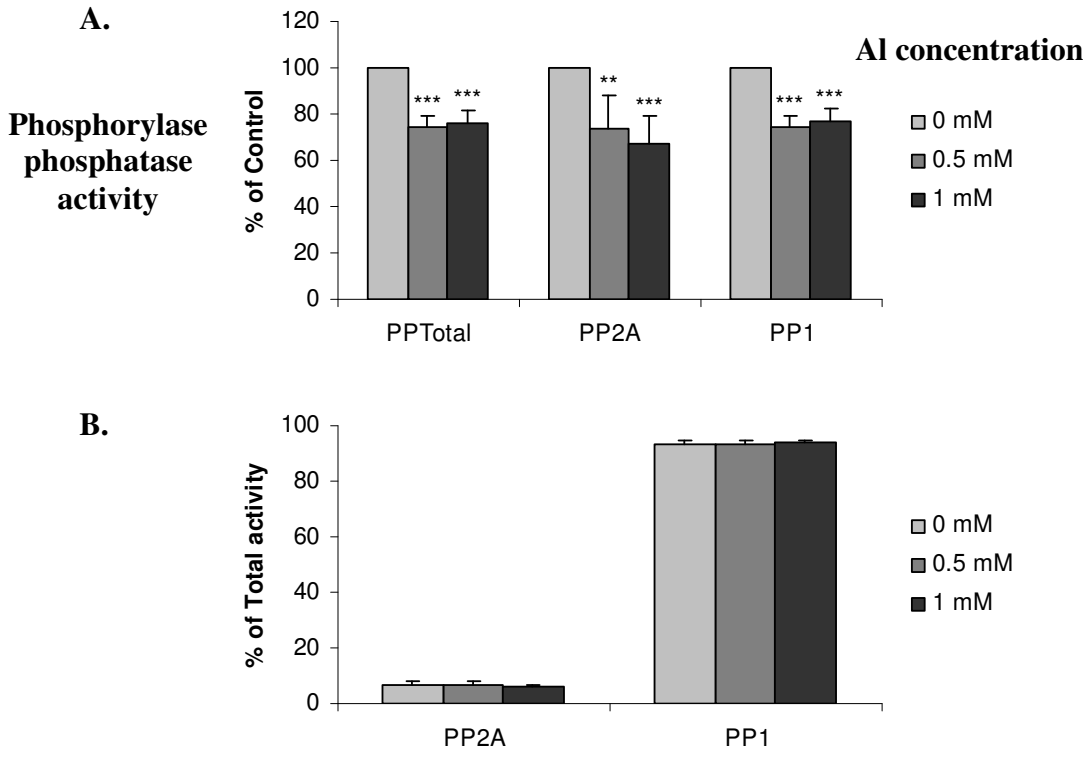

Fig. II.11 – Effect of aluminium on PP1 and PP2A activity in PC12 cells. Cells were incubated with 0.5 mM or 1 mM aluminium for 24 h. The PPtotal, PP1 and PP2A activities were determined using phosphorylase *a* as a substrate. Results obtained are presented as **(A)** percentage of control and **(B)** percentage of total activity. Error bars are mean of duplicates of at least three independent experiments. **, *** Value statistically different from the control $p < 0.01$, $p < 0.001$, respectively.

Cellular PP2A activity was reduced to 73% ± 15% with 0.5 mM $AlCl_3$ and to 68% ± 11.8% with 1 mM $AlCl_3$, whereas for PP1 activity a decrease to 75% ± 4.8% with 0.5 mM $AlCl_3$ and to 77% ± 5.4% with 1 mM $AlCl_3$ was observed. The decrease of about 25% detected for the PP total activity was mainly due to the aluminium induced inhibition of PP1 activity, as shown in Fig. II.11.B. The contribution of PP2A to the total phosphorylase phosphatase activity in PC12 cells was only about 6%, whereas that of PP1 was approximately 94%.

In COS-1 cells (Fig. II.12) the PP total activity decreased to approximately 60% with both 0.5 mM and 1 mM $AlCl_3$.

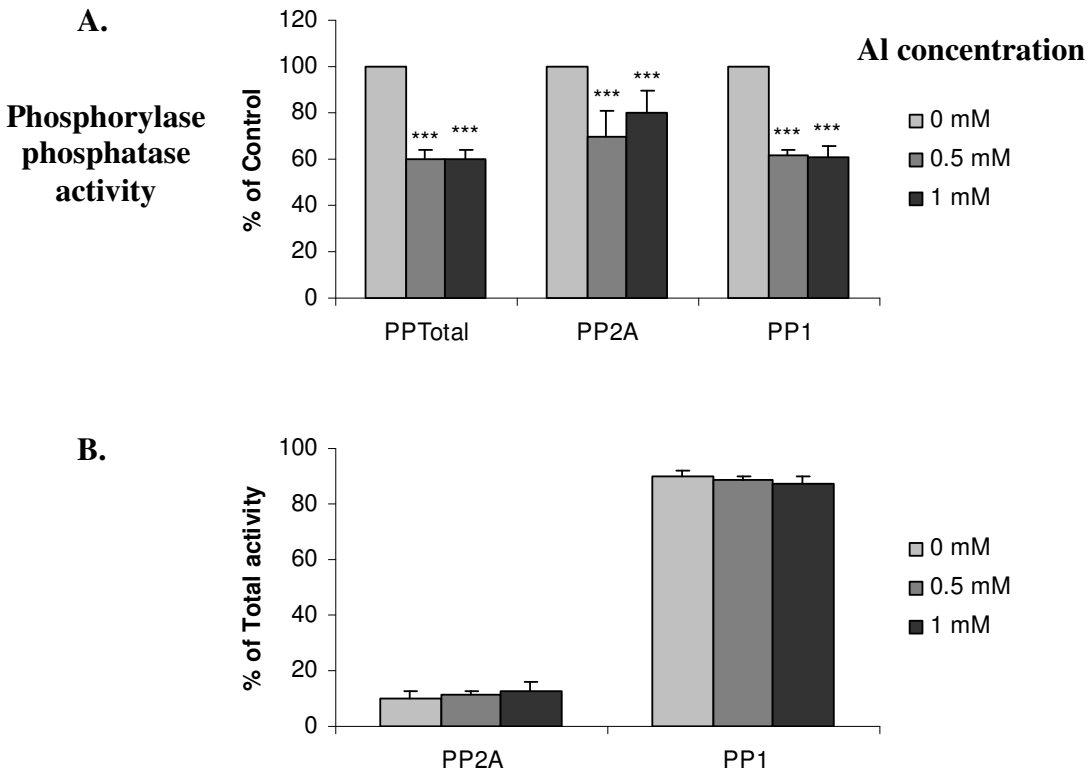

Fig. II.12 – Effect of aluminium on PP1 and PP2A activity in COS-1 cells. Cells were incubated with 0.5 mM or 1 mM aluminium for 24 h. The PP1 and PP2A activity was determined using phosphorylase *a* as substrate. Results obtained are presented as **(A)** percentage of control and **(B)** percentage of total activity. Error bars are mean of duplicates of at least three independent experiments. *** Value statistically different from the control $p < 0.001$.

An inhibition similar to that previously shown for PC12 cells was also observed for COS-1 cells incubated with aluminium. PP2A was inhibited to 70% ± 10.9% and to 80% ± 10.2% respectively by 0.5 mM and 1 mM $AlCl_3$, and PP1 to 61% ±3.1% and to 61% ± 4.6% respectively by 0.5 mM and 1 mM $AlCl_3$. The observed 40% decrease for the total phosphorylase phosphatase activity was mainly due also to PP1 inhibition by aluminium, as verified for PC12 cells. PP2A only contributed with about 10% of the total activity, whereas PP1 accounted for 90%.

II.4. SUMARY OF RESULTS

In this study it was observed that:

- Serum withdrawal alone, in both cell lines, can not account for the observed effects of aluminium.

- Aluminium induced a decrease of cellular viability in both cell lines, both with time of exposure and with the dose.

- Decreased cellular viability was seen to recover in both cell lines, following aluminium withdrawal.

- Aluminium induced a decrease in the expression of both PP1 isoforms, in both cell lines, with time and in a dose dependent manner.

- The decrease induced by aluminium was reverted following aluminium withdrawal, especially in medium with serum.

- The aluminium IC_{50} was similar for both recombinant PP1α and PP1γ_1.

- Aluminium induced a decrease on the PP1 activity *in vivo*, in both cell lines.

This comparative study showed that globally aluminium provoked equivalent effects both in neuronal-like PC12 cells and in non-neuronal COS-1 cells, with similar effects on both PP1 isoforms.

II.5. DISCUSSION

The neurotoxicity of aluminium has been shown to mimic many of the pathophysiological features of AD. Indeed, the accumulation of aluminium in the brain may contribute to the cholinergic deficiency observed in AD patients (Bielarczyk *et al.*, 1998). By potentiating lipid peroxidation, aluminium affects the uptake of choline in nerve terminals (Amador *et al.*, 2001), potentially contributing to the cholinergic dysfunction and neuronal cell degeneration known to occur in AD. In this study, aluminium was shown to decrease PP1 expression levels. Although the precise molecular link between aluminium toxicity and decreased PP1 expression remains to be elucidated, several lines of evidence provide interesting clues. Chronic aluminium exposure impairs long-term potentiation and depression in the rat dentate gyrus *in vivo*, potentially suggesting that aluminium affects both presynaptic and postsynaptic mechanisms of synaptic transmission (Chen *et al.*,

2002). PP1 is not only highly enriched in dendritic spines (Ouimet *et al.*, 1995) and necessary for maintaining long-term depression (Morishita *et al.*, 2001), it was also linked to age-related memory and learning deficits (Genoux *et al.*, 2002). It might be hypothesized that the toxic effect of aluminium may be mediated via its effect on PP1.

In the recovery studies, the decrease in PP1 expression levels was reverted by aluminium withdrawal. This suggests that toxicity induced by low levels of aluminium may be reversible. Recovery effects from aluminium toxicity have been published by either using metal ion chelation or using antioxidants. It was reported that aluminium-induced enhanced expression of glial fibrillary acidic protein was attenuated by some chelators (Yokel and O'Callaghan, 1998). Chronic aluminium exposure through drinking water was shown to induce overexpression of Abeta protein in the hippocampus of rats, but this effect was reverted by the herbal medicine *Dipsacus asper* extract and by vitamin E (Zhang *et al.* 2003). Moreover, it was published that vitamin E protects against aluminium-induced neurotoxicity in rats by reducing the release of proinflammatory cytokines induced by aluminium (Nedzvetsky *et al.*, 2006).

The use of a culture system offers appropriate conditions for continuous inspection of the cells during prolonged periods of exposure to aluminium, along with ability to control aluminium content in the extracellular medium and to examine its uptake into cells (Meiri *et al.*, 1993). It was reported that PC12 cells exposed to $AlCl_3$ exhibited internalized aluminium, measured by atomic absorption spectrometry, linearly proportional to the extracellular aluminium concentration (Bosetti *et al.*, 2001). In this study both cell lines used responded similarly to aluminium exposure. The cellular viability monitored by mitochondrial dehydrogenases activity decreased following aluminium treatment. This finding was in agreement with the decrease in cell viability reported by Suarez-Fernandez *et al.* (1999) following prolonged exposure of cultured astrocytes and neurons to aluminium chloride. In fact, an increasing amount of evidence reported in model studies using cultured cells and animals, has confirmed that aluminium can have a severe neurotoxic effect. It is well known that aluminium has the ability to promote the pro-oxidant properties of transition metals such as iron (Gutteridge *et al.*, 1985; Oteiza, 1994; Xie *et al.*, 1996). It seams that the decrease of MTT reduction following aluminium exposure may reflect modification of mitochondrial function. Alterations in the mitochondrial function may lead to the formation of excess ROS production, which in turn

can lead to oxidative injury. Aluminium accumulation was found significantly increased in the nerve endings, in the presence of an oxidizing system, and a possible role was proposed for aluminium in the promotion and enhancement of oxidant-induced damage believed to occur in neuronal degeneration (Amador *et al.*, 1999). Besides the pro-oxidant effect, aluminium is also known to promote inflammatory events (Campbell *et al.*, 2004; Becaria *et al.*, 2006). Aluminium induced a significant increase in lipid peroxidation and marked elevation of pro-inflammatory cytokines (Nedzvetsky *et al.*, 2006). Platt and co-workers (2001), using histochemical and immunocytochemical studies, suggested that the enhancement of inflammation and the interference with cholinergic projections may be the mode of action through which aluminium causes learning and memory deficits. Additionally, an aluminium impairment of hippocampal long-term potentiation, a model for synaptic plasticity underlying some forms of learning and memory, has been reported in rats both *in vivo* and *in vitro* (Platt *et al.*, 1995).

Under the experimental conditions used, a general decrease of the expression of both PP1 isoforms with time and in a dose dependent manner was observed. However, for low aluminium concentrations, PP1γ_1 expression in COS-1 cells appeared up-regulated, contrary to the down-regulation observed for higher aluminium concentrations. The biological response upon exposure to a chemical can be biphasic. A biphasic response is an increase in response at low-dosage levels and a decrease in response at high-dose levels. Aluminium is capable of producing biphasic effects in diverse cell systems. A high dose of aluminium was observed to decrease protein synthesis, whereas a lower dose increased protein synthesis (Kumar, 1999). Moreover, low aluminium concentrations have been shown to have an activating effect without causing cytotoxicity (Golub *et al.*, 2002). Other experimental conditions, such as brief or prolonged exposure to sodium azide also yielded a similar biphasic effect on PP1γ_1 expression levels (Amador *et al.*, 2004). Besides the decrease of PP1 expression levels, aluminium also induced a reduction of PP1 activity, for both isoforms. The contribution of aluminium to the reduction of endogenous phosphatase activity was previously reported and implicated in the hyperphosphorylation of neurofilament proteins (Shetty *et al.*, 1992). Moreover, following chronic aluminium treatment the levels of protein phosphatases were found to be depleted in the cerebral cortex (Kaur *et al.*, 2006). Indeed, it has been reported that the expression and/or activities of PP1 and other phosphatases are decreased in the affected areas of AD brains (Gong *et*

al., 1993, 1995; Lian *et al.*, 2001; Liu *et al.*, 2005a). Reduced PP1 activity has been related to tau hyperphosphorylation (Baum *et al.*, 1995; Merrick *et al.*, 1997; Iqbal *et al.*, 2000). Decreased PP1α and PP1γ_1 mRNA expression was also reported in samples from AD patients (Mufson *et al.*, 2002). These findings suggest that PP1 activity, protein levels, as well as mRNA levels, are reduced in neurodegeneration. It might be hypothesized that aluminium may contribute to this down-regulation and to that neurotoxicity.

CHAPTER III

EFFECT OF ALUMINIUM ON PRIMARY CORTICAL NEURONAL CULTURES

III. EFFECT OF ALUMINIUM ON PRIMARY CORTICAL NEURONAL CULTURES

III.1. INTRODUCTION

The phosphorylation state of neurofilaments (NF) is altered in neurodegenerative diseases. As NF are confined to the nervous system, they might be one of the best markers reflecting neuronal pathogenic changes seen in some neurological disorders, such as AD. Neuronal accumulation of NF proteins has been seen in a number of neurodegenerative diseases. In the AD brain, in addition to tau, NF are also hyperphosphorylated and accumulated. In fact, the levels of all three neurofilament subunits have been found to be markedly increased and, at least NF-H and NF-M, to be significantly hyperphosphorylated at several sites (Wang *et al.*, 2001; Hu *et al.*, 2002). Evidence points to the involvement of protein phosphatases in the regulation of the phosphorylation state of neurofilaments.

In this chapter a neuronal system (primary cortical cultures) was used to evaluate the effect of aluminium on the expression of both PP1 isoforms (PP1α and PP1γ_1), and on both phosphorylated and nonphosphorylated NF (P-NF and nonP-NF, respectively). The effect of aluminium on the expression of synaptophysin, a 50 kDa protein from synaptic vesicles membrane, was also studied as a nerve terminal marker. A 10 day time course of the same proteins was also performed. The level of expression of those proteins was also evaluated in cortex homogenates from 18 day embryos (E18) and from postnatal rats with 4 (P4) and 7 (P7) days. Time course of protein expression was compared during development in culture and *in vivo*. The aim of this study was to evaluate the effect of aluminium on the expression of proteins in a neuronal system.

III.2. MATERIALS AND METHODS

The complete composition of all solutions and media used, as well as other relevant information, is presented in Appendix I. All reagents were of cell culture grade or ultra pure. Detailed methods are described in Appendix II. Other technical information is also presented in Appendix III.

III.2.1. Rat cortical primary cultures

Rat cortical neurons were dissociated from cortex of Wistar Hannover 18 day rat embryos whose mother was sacrificed by rapid cervical dislocation. Tissues were treated with a solution of 0.45 mg/ml trypsin and 0.18 mg/ml deoxyribonuclease in Ca^{2+}- and Mg^{2+}-free Hank's Balanced Salt Solution (HBSS) for 10 min at 37 ºC. Cells were washed with HBSS supplemented with 10% fetal bovine serum to stop trypsinization, centrifuged at 200 x *g* for 3 min, and further washed and centrifuged with HBSS for serum withdrawal. Cells were pelleted and ressuspended with serum-free Neurobasal medium (Gibco), supplemented with 2% B27 supplement (Gibco), 25 μM glutamate (Sigma-Aldrich), 0.5 mM L-glutamine (Gibco), 60 μg/ml gentamicine (Gibco) and 0.001% Phenol Red (Sigma-Aldrich) (complete Neurobasal medium). Viability and cellular concentration were assessed using Trypan Blue exclusion (0.4% Trypan Blue solution, Sigma-Aldrich). Blue stained cells (dead) or unstained (living) were counted in a haemocytometer chamber, with cellular viability normally being greater than 95%. These neuronal cells were finally plated at a density of $0.9x10^6$ cells/well, in complete Neurobasal medium in poly-D-lysine (0.1mg/ml) pre-coated 6-well plates, and grown at 37 ºC in an atmosphere of 5% CO_2. Seven days after plating, 500 μl of cultured medium was substituted by 500 μl of Neurobasal medium with B27, L-glutamine but lacking glutamate.

III.2.2. Time course of protein expression

For an evaluation of the time course of PP1 (PP1α and PP1γ_1), neurofilaments (NF) (phosphorylated and nonphosphorylated) and synaptophysin expression in primary

cultures, cortical neurons isolated and plated on 6-well plates at a density of $0.9x10^6$ cells/well in complete Neurobasal medium were incubated at 37 ºC and allowed to grow and differentiate for 1, 3, 5, 7, 10 and 11 days *in vitro* (DIV). Extracts from the cells collected at 11 DIV were used as the control of 10 days time course. At the specified time points (1 to 11 DIV), the cortical neurons were washed with PBS and harvested in boiling 1% SDS. Cell lysates were boiled for 10 min and sonicated for 30 sec, and the total protein concentration in each sample was determined using the BCA protein assay kit (see Appendix II). Samples were separated by 7.5% SDS-PAGE and immunoblotting was performed with antibodies CBC2C (anti-PP1α), CBC3C (anti-PP1γ1), SMI31 (anti-P-NF), SMI32 (anti-nonP-NF) or anti-synaptophysin. Anti-β-tubulin was also used as a control. The dilutions and the secondary antibodies used are presented in Table III.1. Detection and analysis was performed as described previously in Chapter II.

For this study, samples from isolated cortex of 18 day embryos (E18) and from cortex of postnatal rats with 4 and 7 days of life (P4 and P7) were also used. Those samples were collected and processed as previously described.

Table III.1 List of antibodies used.

Target Protein	Primary Antibody Assay/Dilution	Secondary Antibody Assay/Dilution
PP1α	CBC2C IB dilution: 1:2500	Horseradish Peroxidase conjugated Rabbit IgG IB dilution: 1:5000
PP1γ_1	CBC3C IB dilution: 1:2500	Horseradish Peroxidase conjugated Rabbit IgG IB dilution: 1:5000
Phosphorylated NF	SMI31 IB dilution: 1:1000	Horseradish Peroxidase conjugated Mouse IgG IB dilution: 1:5000
Nonphosphorylated NF	SMI32 IB dilution: 1:1000	Horseradish Peroxidase conjugated Mouse IgG IB dilution: 1:5000
Synaptophysin	Anti-Synaptophysin IB dilution: 1:7500	Horseradish Peroxidase conjugated Mouse IgG IB dilution: 1:5000
β-Tubulin	Anti-β-Tubulin IB dilution: 1 μg/ml	Horseradish Peroxidase conjugated Mouse IgG IB dilution: 1:5000

IB – immunoblot.

III.2.3. Exposure of cortical neurons to aluminium

To study the effect of aluminium on the protein expression in cortical neurons, a set of plates were prepared as described above. After 10 days in culture, cells were washed with serum-free and phosphate-free DMEM. Cells were then incubated in this medium with $AlCl_3$ at final concentrations of 0.01 mM and 0.05 mM for 24 h at 37 ºC. A control of cells, washed and incubated only with serum-free and phosphate-free DMEM for 24 h, was also performed. Cells with 11 DIV maintained in Neurobasal medium (NB) were kept as control for medium substitution as well. The cortical neurons were washed with PBS, harvested in boiling 1% SDS and processed as described above to perform the immunodetection of PP1, NF and synaptophysin. The cellular viability was also determined with the MTT assay (Mossman, 1983), as previously described in Chapter II.

III.3. RESULTS

III.3.1. Time course of protein expression

An evaluation of the time course of protein expression was performed in primary cortical cultures from 1 DIV to 11 DIV. The proteins analysed were both PP1 isoforms (PP1α and PP1γ_1), both phosphorylated and nonphosphorylated NF proteins, and synaptophysin. The levels of expression of these proteins were also evaluated in cortex homogenates from 18 days embryos (E18), and postnatal day 4 (P4) and 7 (P7) rats. Data is presented as percentage of control, with 100% of expression being defined as the level of protein at 11 DIV.

III.3.1.1. Time course of PP1α and PP1γ_1 expression

The expression of PP1α and PP1γ_1 increased with time in culture in an analogous way for both proteins, as presented on Fig. III.1. For PP1α the values of expression were 41% ± 6.6% for 1 DIV, 55% ± 7% for 3 DIV, 73% ± 6.7% for 5 DIV, 94% ± 4.4% for 7 DIV and 103% ± 4% for 10 DIV. The expression of PP1γ_1 achieved 49% ± 4.4% at 1

DIV, 58% ± 4.3% at 3 DIV, 61% ± 4% at 5 DIV, 75% ± 5.2% at 7 DIV and 97% ± 9.6% at 10 DIV. At 10 DIV both PP1 isoforms reached values of expression similar to the control (expression at 11 DIV).

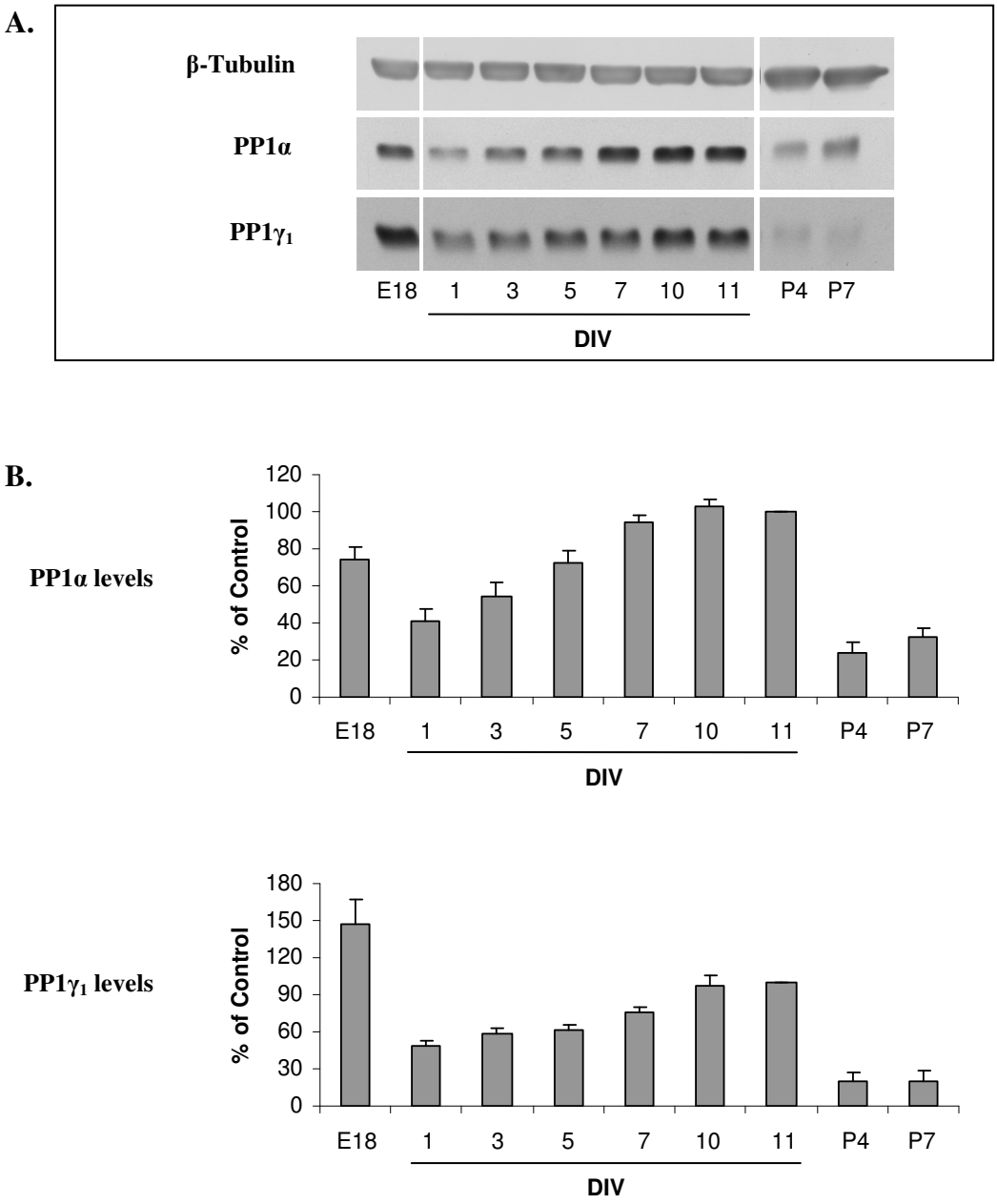

Fig. III.1 – Time course of PP1α and PP1γ_1 expression in primary cortical neurons. Cells were collected at different days in culture (1 to 11) and analysed. Samples from cortex of 18 days embryos (E18) and from rats with 4 (P4) and 7 (P7) days were also analysed. The expression levels of PP1α and PP1γ_1 were analysed by immunobloting with specific antibodies **(A)**. β-Tubulin levels were also assessed as a control. Results obtained from at least three independent experiments were quantified and are represented graphically **(B)** as percentage of control (expression at 11 DIV) and expressed as mean ± SEM.

The levels of expression obtained from E18 samples were 74% ± 6.9% for PP1α and reached 148% ± 19.6% for PP1γ_1. From P4 and P7 samples the expression values were quantified as being 24% ± 6.1% and 33% ± 4.1% for PP1α, and 20% ± 7.2% and 20% ± 7.7% for PP1γ_1. Overall the expression of both PP1 isoforms was analogous in both samples, with expression levels decreasing dramatically on the first day of culture compared to the E18 samples, especially for PP1γ_1, and thereafter recovering with time in culture up to 10-11 DIV. Following birth, both PP1α and PP1γ_1 levels decreased again, as see for the P4 and P7 samples.

III.3.1.2. Time course of P-NF and nonP-NF expression

The antibody anti-P-NF (SMI31) reacts with high molecular weight neurofilament (NF-H) and, to a lesser extent, with medium molecular weight neurofilament (NF-M). NF-H is 200 KDa and NF-M is 160 KDa, and therefore both proteins were quantified separately. The antibody anti-nonP-NF (SMI32) reacts only with NF-H but detects two bands of 200 and 180 KDa, both bands were quantified together.

The expression of both phosphorylated, NF-H and NF-M, increased with time of culture, as well as the expression of the nonphosphorylated NF (Fig.III.2). The expression levels of NF-H (phosphorylated) were 17% ± 11.7%, 55% ± 18.7%, 83% ± 11.6%, 90% ± 7.5% and 97% ± 4.9% for 1, 3, 5, 7 and 10 DIV, respectively. And for NF-M (phosphorylated) were 3% ±1.4%, 16% ± 7%, 45% ± 11.1%, 69% ± 13% and 94% ± 12.9%, respectively. For the same time points the expression of nonphosphorylated NF reached 35% ± 6.2%, 48% ± 5.9%, 69% ± 6%, 84% ± 15.3% and 99% ± 3.7%. At the 10th day of culture the expression of both P-NF and nonP-NF yielded values similar to the control (expression at 11 DIV). The values of E18, P4 and P7 samples were 26% ± 19.1%, 7% ± 2.6% and 5% ± 2.8% for NF-H (phosphorylated), 66% ± 28%, 29% ± 8.4% and 21% ± 8.3% for NF-M (phosphorylated) and 61% ± 15.1%, 142% ± 24.5% and 27% ± 7.3% for nonP-NF, respectively.

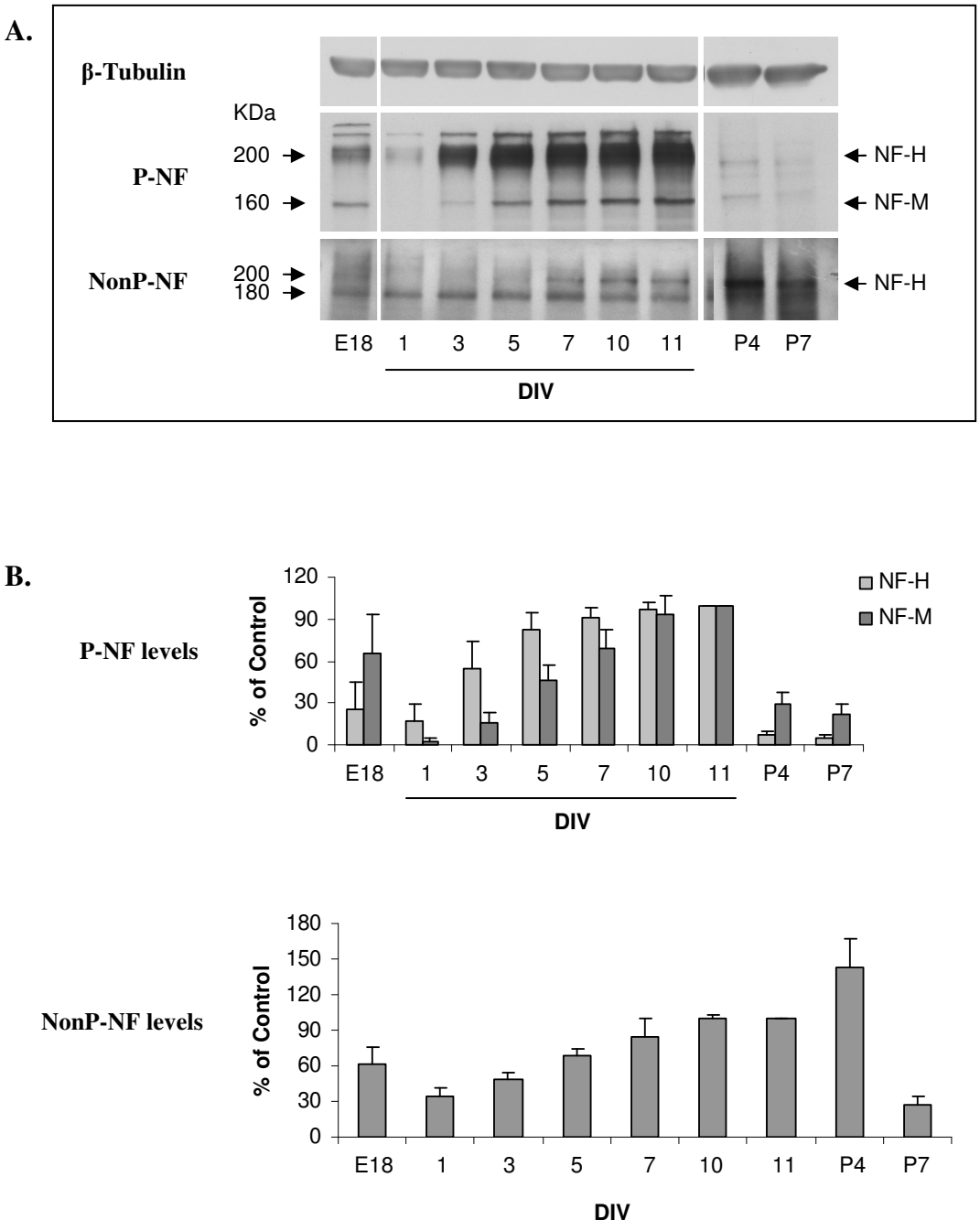

Fig. III.2 – Time course of neurofilament expression in primary cortical neurons. Cells were collected at different days in culture (1 to 11) and analysed. Samples from cortex of 18 days embryos (E18) and from rats with 4 (P4) and 7 (P7) days were also analysed. The expression level of neurofilaments phosphorylated **(P-NF)** and nonphosphorylated **(nonP-NF)** were analysed by immunobloting with specific antibodies **(A)**. β-Tubulin levels were also assessed as a control. Results obtained from at least three independent experiments were quantified and are represented graphically **(B)** as percentage of control (expression at 11 DIV) and expressed as mean ± SEM.

III.3.1.3. Time course of synaptophysin expression

The expression of synaptophysin, a synaptic vesicle protein used as a marker, increased gradually with time in culture (Fig.III.3). Starting only with 3% ± 1.3% at 1 DIV, increased to 13% ± 3.5%, 36% ± 4.8%, 57% ± 4.8% and 90% 2.5% at 3, 5, 7 and 10 DIV, respectively. Only trace levels were detected in E18 samples, but levels similar to control were detected in the postnatal samples (73% ± 4.5% for P4 and 87% ± 6.5% for P7).

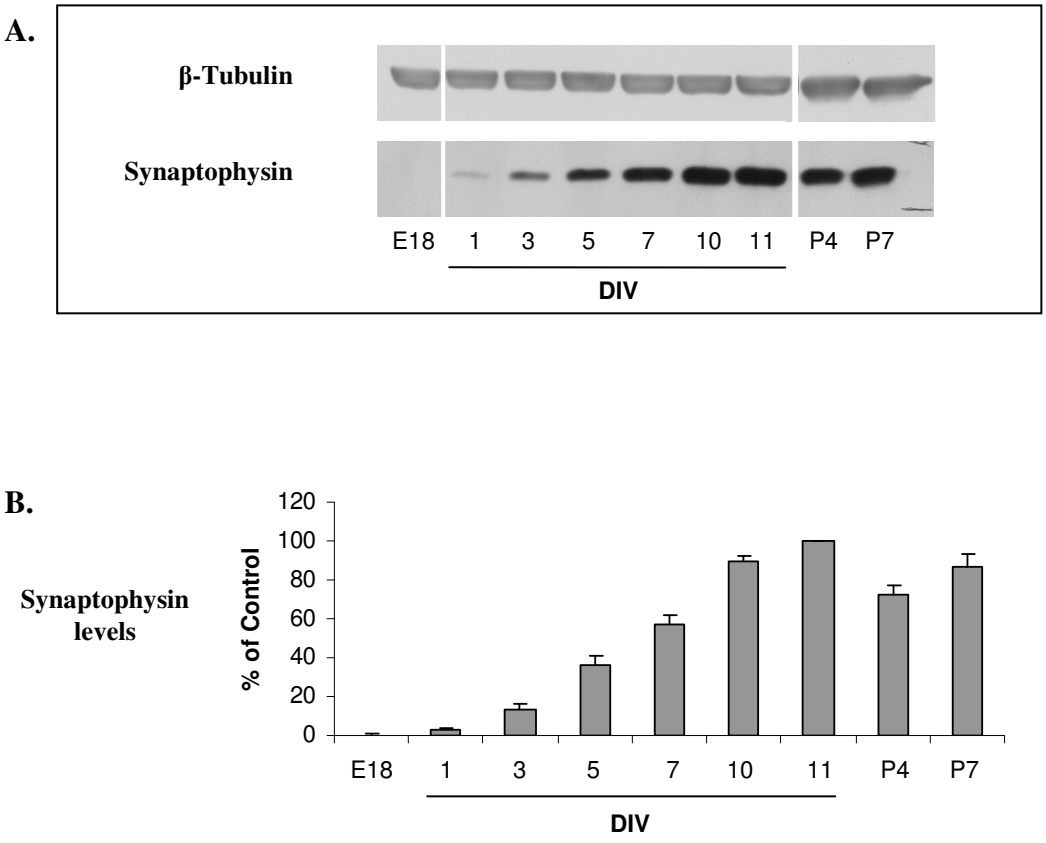

Fig. III.3 – Time course of synaptophysin expression in primary cortical neurons. Cells were collected at different days in culture (1 to 11) and analysed. Samples from cortex of 18 days embryos (E18) and from rats with 4 (P4) and 7 (P7) days were also analysed. The expression level of synaptophysin was analysed by immunobloting with specific antibody **(A)**. β-Tubulin levels were also assessed as a control. Results obtained from at least three independent experiments were quantified and are represented graphically **(B)** as percentage of control (expression at 11 DIV) and expressed as mean ± SEM.

III.3.2. Effect of aluminium on cellular viability

The viability of cortical neurons was evaluated following aluminium exposure for 24 h. A significant decrease to 50% ± 12% was observed, but only with the higher aluminium concentration (0.05 mM) as shown in Fig. III.4. The lower concentration, 0.01 mM aluminium, did not affect significantly the viability of cortical neurons, yielding 90% ± 4.5% in relation to the control (cells incubated in serum-free, phosphate-free DMEM for 24 h in the absence of aluminium). However, a significant decrease of 34% ± 9% in cell viability had already been induced by the change in medium from Neurobasal to serum-free, phosphate-free DMEM.

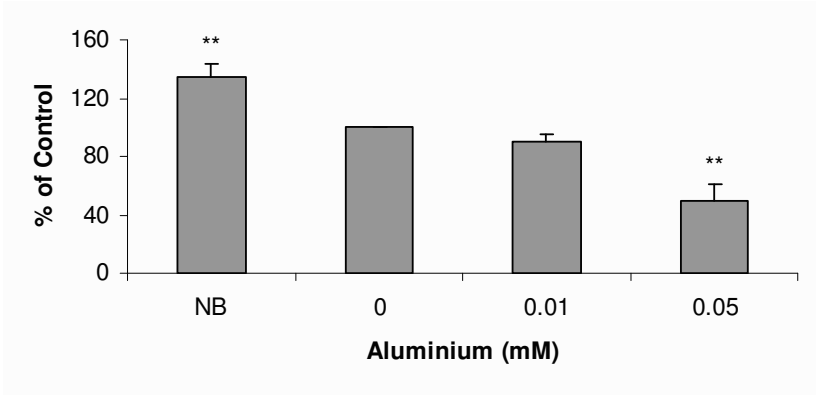

Fig. III.4 - Effect of aluminium on cellular viability in primary cortical neurons. Cells with 10 days in culture were incubated with 0.01 mM or 0.05 mM aluminium for 24 h. Cells with 11 DIV in Neurobasal medium (NB) were included for comparison. Results are expressed as percentage of control (absence of aluminium) as mean ± SEM of triplicate determinations from at least three independent experiments. ** Value statistically different from the control ($p < 0.01$).

III.3.3. Aluminium effect on protein expression

The effect of aluminium on the expression of both PP1 isoforms, P-NF, nonP-NF and synaptophysin was evaluated. Data is presented as percentage of control (cells incubated in serum-free, phosphate-free DMEM in the absence of aluminium). Statistical differences are presented in relation to the control.

III.3.3.1. Effect of aluminium on PP1α and PP1γ_1 expression

No significant changes on the expression of both PP1α and PP1γ_1 were observed following aluminium exposure, as shown on Fig. III.5. The expression of PP1α was at 103% ± 6.8% and 104% ± 9.1%, after exposure to 0.01 mM and 0.05 mM $AlCl_3$ for 24 h. Similar values were also observed for PP1γ_1 (97% ± 7.8% and 110% ± 8.9% after incubation with 0.01 and 0.05 mM $AlCl_3$, respectively). However, the expression of both isoforms decreased significantly by 41% ± 9% for PP1α and 30% ± 7.2% for PP1γ_1 due to the medium change from Neurobasal to serum-free, phosphate-free DMEM.

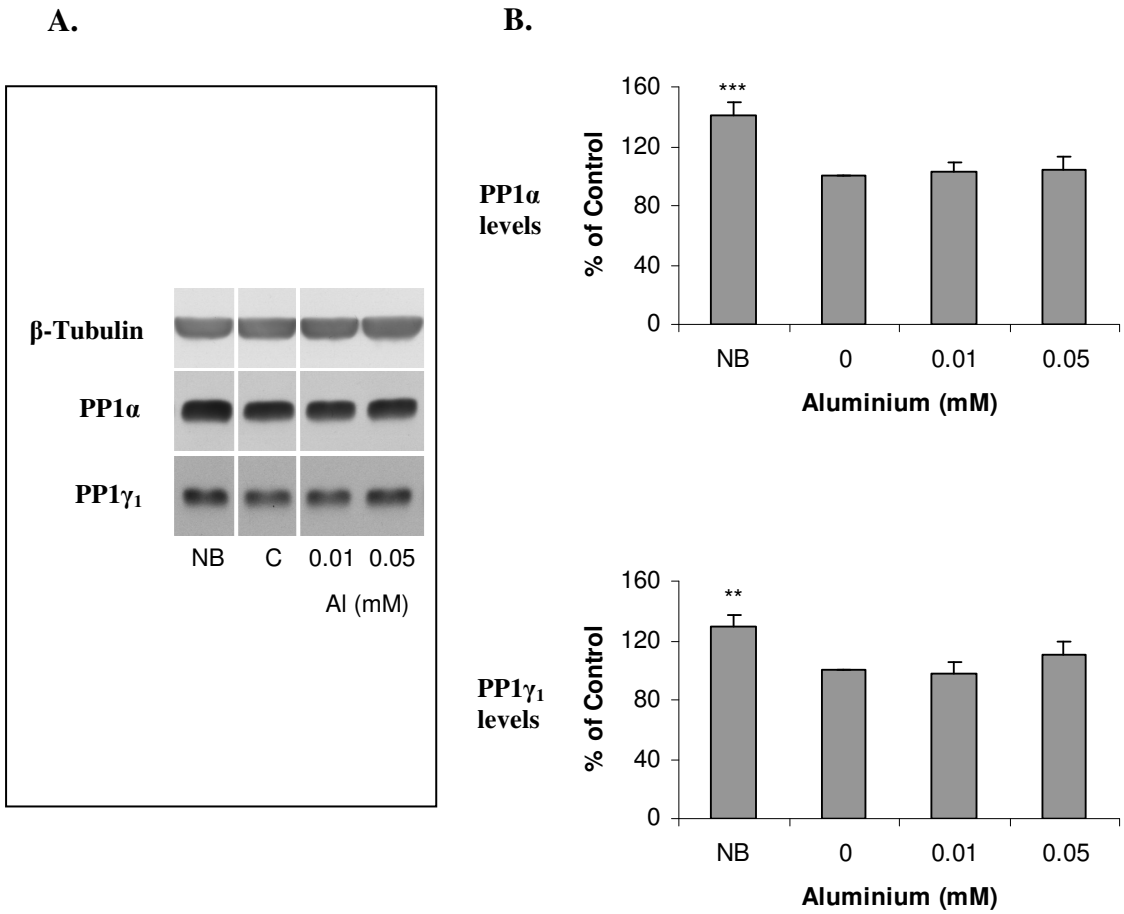

Fig. III.5 - Effect of aluminium on PP1 isoform expression in primary cortical neurons. Cells with 10 days in culture were incubated with 0.01 mM or 0.05 mM aluminium in serum-free phosphate-free DMEM for 24 h. Cells with 11 DIV in Neurobasal medium (NB) were included for comparison. The expression level of PP1α and PP1γ_1 were analysed by immunobloting with specific antibodies **(A)**. β-Tubulin levels were also monitored as a control. Results obtained from at least three independent experiments were quantified and are represented graphically **(B)** as percentage of control (absence of aluminium) and expressed as mean ± SEM.

III.3.3.2. Aluminium effect on P-NF and nonP-NF expression

As previously described, phosphorylated NF-H and NF-M were quantified separately; on the other hand, both nonphosphorylated NF-H isoforms were quantified together. The higher aluminium concentration (0.05 mM) induced the virtual disappearance of both phosphorylated NF-H and NF-M (1% ± 1.3% and 0%, respectively) and a significant reduction of nonP-NF expression to 7% ± 3.3% (Fig. III.6). In contrast, the lower aluminium concentration (0.01 mM) had no effect on the expression of both phosphorylated NF-H and NF-M (91% ± 9.4% and 85% ± 12.9%, respectively), but induced a significant decrease of nonP-NF expression to 57% ± 11.2%. Cells with 11 DIV in Neurobasal medium (NB) globally presented no significant differences in the expression levels of both phosphorylated NF-H and NF-M (115% ± 13.9% and 143% ± 50.24%, respectively) or of nonP-NF (107% ± 31.1%).

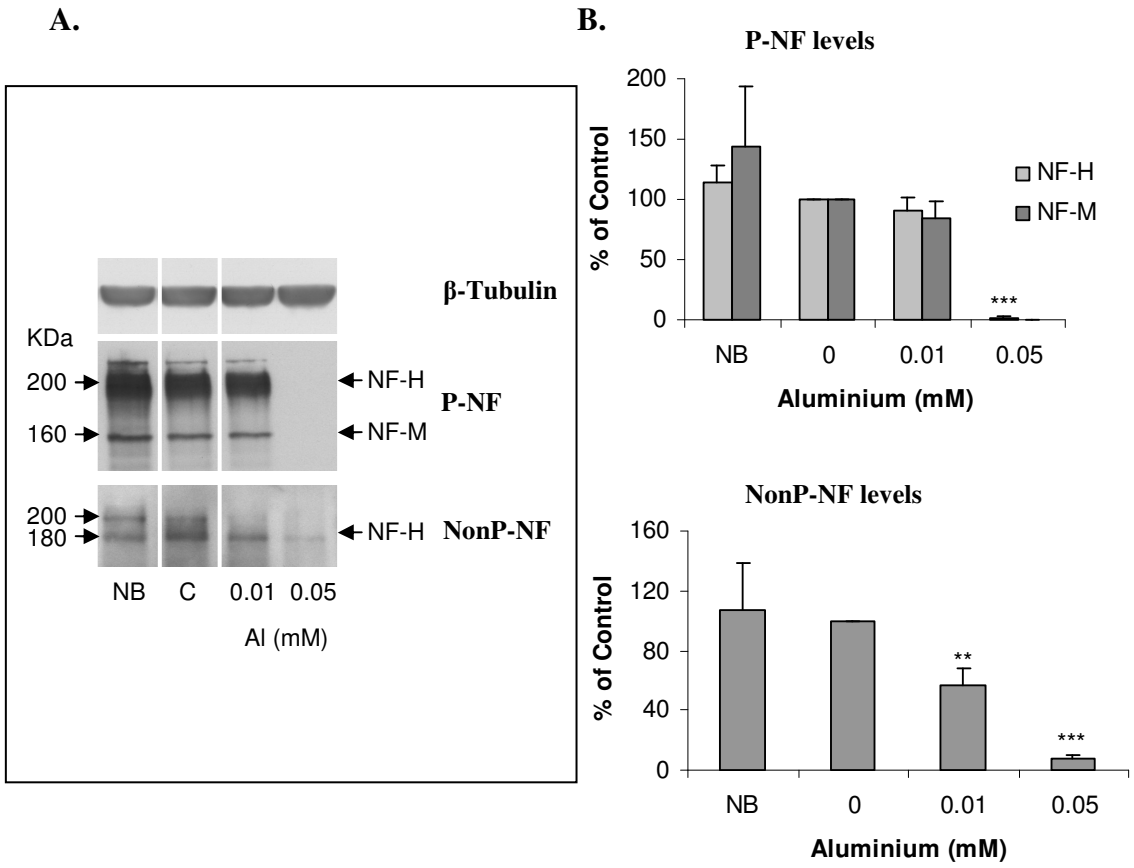

Fig. III.6 - Effect of aluminium on neurofilament expression in primary cortical neurons. Cells with 10 days in culture were incubated with 0.01 mM or 0.05 mM aluminium in serum-free phosphate-free DMEM

for 24 h. Cells with 11 DIV in Neurobasal medium (NB) were included for comparison. The expression level of phosphorylated **(P-NF)** and nonphosphorylated **(nonP-NF)** neurofilaments were analysed by immunobloting with specific antibody **(A)**. β-Tubulin levels were also assessed as a control. Results obtained from at least three independent experiments were quantified and are represented graphically **(B)** as percentage of control (absence of aluminium) and expressed as mean ± SEM (**, *** value statistically different from the control $p < 0.01$, $p < 0.001$, respectively).

III.3.3.3. Effect of aluminium on synaptophysin expression

The expression of synaptophysin only decreased significantly following exposure to 0.05 mM $AlCl_3$ for 24 h (88% ± 3.5% of the control). Incubation with 0.01 mM $AlCl_3$ did not induce changes on this protein expression (97% ± 3%), as presented in Fig. III.7. Also, cells with 11 DIV in Neurobasal medium exhibited synaptophysin expression levels similar to the control (94% ± 4.9%).

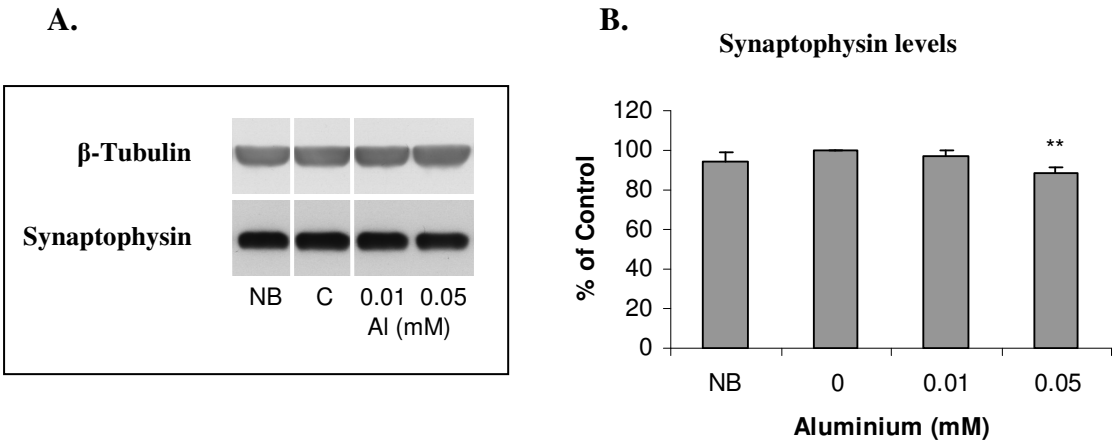

Fig. III.7 - Effect of aluminium on synaptophysin expression in primary cortical neurons. Cells with 10 days in culture were incubated with 0.01 mM or 0.05 mM aluminium in serum-free phosphate-free DMEM for 24 h. Cells with 11 DIV in Neurobasal medium (NB) were included for comparison. The expression level of synaptophysin was analysed by immunobloting with specific antibody **(A)**. β-Tubulin levels were also assessed as a control. Results obtained from at least three independent experiments were quantified and are represented graphically **(B)** as percentage of control (absence of aluminium) and expressed as mean ± SEM (** value statistically different from the control $p < 0.01$).

III.4. SUMARY OF RESULTS

In this study it was observed that:

- All proteins studied (both PP1 isoforms, both NF proteins and synaptophysin) increased their expression during the 10 day time course in primary cortical neurons.

- Only the higher aluminium concentration (0.05 mM) induced a decrease in cellular viability. However, 11 DIV cells in Neurobasal medium exhibited cellular viability values higher than the control.

- The expression of both PP1 isoforms remained largely unchanged after aluminium exposure. However, higher expression levels of both PP1 isoforms were observed in 11 DIV cells in Neurobasal medium, compared to the control.

- Both aluminium concentrations induced a decrease in the expression of nonP-NF.

- The higher aluminium concentration (0.05 mM) abolished both medium and high molecular weight P-NF expression and decreased synaptophysin expression.

- On the other hand, no significant differences in the expression levels of neurofilaments or synaptophysin were observed in 11 DIV cells in Neurobasal medium.

III.5. DISCUSSION

The primary culture system of dissociated neurons provides a useful tool that enables the observation of pharmacological, functional and morphological changes induced by a variety of agents, including aluminium. Cultured neurons extend neurites, form synaptic contacts, show electrophysiological activities, can be maintained more than 1 month, and exhibit morphological maturation of synapses during the culture period. These features of cultured neurons are similar to those of neurons *in vivo* (Ichikawa *et al.*, 1993). In this work the studied proteins were monitored with time to determine the time course of their expression. The expression of all proteins analysed increased with time, denoting the good conditions of the culture and thus conducting to its maturation. This observation was particularly important for synaptophysin expression, since this protein is the main protein of synaptic vesicles and hence a good marker for monitoring synaptogenesis. Ichikawa *et al.* (1993) observed an increase in the number of synaptic vesicles per terminal with DIV,

which was correlated with the increase verified in synaptophysin expression with DIV. During cortical neuron maturation an increase in the expression levels of synaptophysin was observed during the first 15-20 days and then remained stable through 60 DIV (Lesuisse and Martin, 2002). Also, increasing expression of NF with time may be correlated with the growth of neuronal processes and therefore with culture maturation.

In this chapter, the aluminium concentrations used were lower than those used in the previous studies, since cellular viability tests revealed cortical neurons to be more sensitive to aluminium than the cell lines previously used. The previous concentration used (0.5 and 1 mM) induced high cytotoxicity to neurons, disrupting neurons and causing cells to die. Thus, the lower concentrations used (0.01 and 0.05 mM) were determined to induce low and mild cytotoxicity levels. However, these lower aluminium concentrations seemed not to be sufficient to induce a decrease in PP1 expression. The expression of both PP1 isoforms was not affected by aluminium. However, a decrease of synaptophysin and NF expression was observed following aluminium treatment. Kawahara and co-workers (1992) also reported an impairment of synapse formation in cultured neurons of rat cerebral cortex following long-term aluminium exposure. It was also reported that the *in vitro* formation of synaptic vesicles from PC12 membranes, containing the protein synaptophysin, was inhibited by aluminium (Cleves *et al.*, 1997). The aluminium-induced decrease of synaptophysin expression levels was correlated with the decrease verified on cellular viability. This suggests that the observed decrease of synaptophysin expression is due to an increase of neuronal disruption induced by aluminium, especially on axons and nerve terminals. Also, the decrease of both NFs levels might be due to the aluminium induced axon disruption. The higher aluminium concentration induced a decrease of cellular viability, monitored by assaying mitochondrial enzyme activity, this suggesting the occurrence of mitochondria impairment induced by aluminium. Mitochondria impairment is one of the factors that contribute to neurodegeneration. Indeed, reduced expression of NF mRNA was found in human neurodegenerative diseases (AD and ALS) (Crapper *et al.*, 1988; Bergeron *et al.*, 1994), as well as in aged animals (Parhad *et al.*, 1995). Additionally, Munirathinam *et al.* (1996) observed that primary cortical cultures derived from newborn rats that were exposed on 6 DIV to 0.1-1 mM $AlCl_3$, after 48 h of aluminium exposure, many nerve cell bodies were swollen; a beading of neurites and a disruption of the neuritic network were also observed suggesting neurodegeneration.

In this study, using primary neuronal cultures, aluminium had no effect on the expression of both PP1 isoforms, in contrast to the aluminium-induced down-regulation seen in both cell lines used in the previous chapter. These results may be explained by the different susceptibilities of the cells used. In fact, glial cells have been reported to be a primary target of aluminium, rather than neurons. Following aluminium treatment under the same conditions, oxidative events were observed in glioma cells, but no significant changes were observed in neuroblastoma cells, suggesting that glia may have a higher susceptibility to aluminium than neurons (Campbell *et al.*, 1999). It was reported that long exposure to aluminium induced accumulation of the metal both in neurons and astrocytes (Suarez-Fernandez *et al.*, 1999). Interestingly, aluminium neurotoxicity occurred in neuroglial cultures containing approximately 10% astrocytes but not in near-pure neuronal cultures containing only 1% astrocytes. Apoptotic condensation and fragmentation of chromatin, found in aluminium-treated astrocytes but not in co-cultured neurons, suggests that aluminium can induce the apoptotic degeneration of astrocytes and that this toxicity is critical to neuronal degeneration and death (Suarez-Fernandez *et al.*, 1999). Moreover, extended impairment of astrocytic function following aluminium injection either intraperitoneally or stereotactically into the lateral cerebral ventricles of rats, suggests that these cells may be the primary targets of aluminium neurotoxicity (Guo-Ross *et al.*, 1999).

On the other hand, besides the good culture maturation verified by the time course experiments, the medium substitution from B27-suplemented Neurobasal medium to serum-free, phosphate-free DMEM required for the aluminium experiments, induced some alterations in the culture. These changes were evaluated by the differences verified between the 11 DIV cells in Neurobasal (used as the control of the time course experiments) and the cells with 10 DIV in Neurobasal plus 24h in serum-free, phosphate-free DMEM without aluminium (the control of aluminium experiments). A decrease in the cellular viability was observed, as well as a decrease in the expression levels of both PP1 isoforms due to the medium substitution. However, the medium substitution did not influence the expression levels of neurofilaments and of synaptophysin which did not alter; they only decreased following aluminium exposure. The medium substitution induced different effects on different protein expression levels, probably because different mechanisms were involved conducting to this result. Immunocytochemical studies were also intended to be performed to assess the aluminium induced alterations on the

localization of those proteins and eventually apoptosis. However, the entire medium substitution, from B27- supplemented Neurobasal to serum-free, phosphate-free DMEM, induced by itself changes in cell morphology that prevented these studies for being performed. The medium exchange seemed to be injurious to the neuronal culture, probably due to the withdrawal of essential neurotrophic factors that were excreted to the medium during neuronal maturation or due to differences between media composition that compromised the culture. In fact, B27-supplemented Neurobasal medium with lower osmolarity than DMEM medium and reduced cysteine and glutamine concentrations, led to higher survival of hippocampal neurons (Brewer *et al.*, 1993).

CHAPTER IV

SELDI-TOF MS ANALYSIS OF ALTERED ALUMINIUM-INDUCED PROTEOMIC PROFILING

IV. SELDI-TOF MS ANALYSIS OF ALTERED ALUMINIUM-INDUCED PROTEOMIC PROFILING

IV.1. INTRODUCTION

Aluminium may be implicated in the alteration of the expression of proteins other than those previously studied. In this chapter we analysed the alteration in proteomic profiling induced by aluminium using *in vitro* and *in vivo* systems. The *in vitro* system used consisted of two cell lines (PC12 and COS-1) treated with aluminium. The *in vivo* analysis was carried out with mice injected with aluminium directly in the brain.

For proteomic evaluation, the ProteinChip technology and surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry (MS) (Ciphergen Biosystems) was used. ProteinChip technology coupled with SELDI-TOF MS is an effective tool that allows performing the separation and detection of the relative expression levels of proteins directly from biological samples under different conditions. The system consists of three major components: the ProteinChip Array, the chip reader and the software, which allows the capture, purification, analysis and processing of individual proteins of interest from complex biological mixtures. The ProteinChip System detects and accurately calculates the mass of compounds based on measured time-of-flight.

Protein discovery from the aluminium treated and control samples was performed by mass measurement, the ProteinChip Software provided a graphical comparison of multiple retentate maps allowing to identify proteins that were differentially expressed in the presence of aluminium.

IV.2. MATERIALS AND METHODS

The complete composition of all solutions and media used, as well as other relevant information, is presented in Appendix I. All reagents were of cell culture grade or ultra

pure. Detailed methods are described in Appendix II. Detailed chip characterization and other technical information are also presented in Appendix III.

IV.2.1. *In vitro* and *in vivo* sample processing

IV.2.1.1. PC12 and COS-1 sample preparation

To evaluate the effect of aluminium on protein expression *in vitro*, two cell lines were used. PC12 and COS-1 cells were grown on 6-well plates, until 80% confluency was achieved, in complete RPMI or complete DMEM, respectively. Cells were washed twice with serum-free and phosphate-free medium and incubated with $AlCl_3$ solution at final concentration of 0.5 or 1 mM (in serum-free and phosphate-free medium) for 24 or 48 h. Control cells incubated with phosphate-free medium with serum (10% HS and 5% FBS for PC12 or 10% FBS for COS-1 cells, "with serum") or serum-free and phosphate-free medium ("without serum") for the same periods of time were also prepared. After incubation, cells were washed with PBS, harvested in 1 ml PBS, centrifuged at 310 x *g* for 5 min at 4 ºC and the pellet stored at -80 ºC. Pellets were homogenized in 50 µl PBS/0.5% octyl glucopyranoside (OGP) solution and sonicated for 20 sec. Protein concentration was estimated using the BCA protein assay and adjusted to 3 µg/µl with PBS/0.5% OGP solution in a final volume of 40 µl (120 µg of total protein). Samples were diluted 1:3 in the respective binding buffers for each chip type used.

IV.2.1.2. Mouse sample preparation

To evaluate the effect of aluminium on protein expression *in vivo*, C57BL/6J mice were used. A group of mice, females with 9 months, were weighted and anaesthetized with Ketamine 150 mg/Kg (injected intramuscularly) and Xylazine 10 mg/Kg (injected intraperitoneally). A few minutes later, a small incision in the skin from their head was performed and they were injected with one dose of aluminium lactate solution (1 µl containing 200 µg aluminium or 680 pmoles, 0.68 mM) in the left frontoparietal cortex area (1.5 mm deep). Control groups of mice injected with saline solution (Saline) or punctured with a needle (Sham) in the same cortex area were also performed. Each group

consisted of at least four animals. After injection, the skin was stapled and the animals were allowed to recover for 3 h or 24 h before being sacrificed by decapitation. The cortex was removed and area surrounding the puncture (4-5 mm^3) was isolated, frozen in dry ice and stored at -80 ºC. All samples were homogenized in 50 µl PBS/0.5% OGP and sonicated for 20 sec. Protein concentration was estimated with the BCA protein assay and adjusted to 3 µg/µl in PBS/0.5% OGP solution in a final volume of 40 µl (120 µg of total protein). Samples were diluted 1:3 in the respective binding buffers for each type of chip used.

IV.2.2. ProteinChip array analysis

In this work, chips with different matrices (precoating) were used (ProteinChip Arrays, Ciphergen Biosystems), namely: strong anion-exchange (SAX2), weak cation-exchange (WCX2), reverse phase (H4), normal phase (NP20) and immobilized affinity capture (IMAC3). The coating "enhances" the surface to bind preferentially a particular class of proteins based on their physicochemical properties. Each chip has appropriate binding and washing buffers that are used to equilibrate the chips, to dilute samples and to wash after incubation (for details see Appendix II). The use of chips with different coating and treated with different buffers with different pH allows sample proteins to bind to the chip matrix with different affinities, depending on their pI (isoelectric point). An assortment of chips and buffers allows to identify a wide range of proteins.

IV.2.2.1. Bioprocessor method

Chips were equilibrated (pre-washed) with 10 ml of the respective binding and washing buffer, in a 15 ml sterile tube, with vigorous shaking for 5 min. Chips were removed from the tubes, allowed to air dry and assembled in the bioprocessor (Ciphergen Biosystems) (see Appendix II). Samples (5 µl), diluted in the correspondent binding and washing buffer (1µg/µl), were loaded into appropriate wells and incubated for 90 min at RT with vigorous shaking. After incubation a quick wash was performed by pipetting 100 µl/well of the respective binding and washing buffer. The buffer was removed and the chips were also removed from the bioprocessor. Chips were washed with 10 ml of binding and washing buffer three times for 5 min each in a 15 ml conical centrifuge tube with

vigorous shaking. They were briefly rinsed with 10 ml of HPLC grade water and allowed to air dry. Fresh EAM (Energy Absorbing Molecule) solution (see Appendix I) was loaded in each spot (2 µl/spot) and allowed to air dry for 10 min, this caused the entire mixture to crystallize as it dried. Chips were then analyzed by SELDI-TOF MS as described below.

IV.2.2.2. Direct spot method

A NP20 chip was loaded with known molecular weight peptide and protein standards. The chip was washed with HPLC water and incubated with 1 µl/spot of a mixture of peptides or protein standards and 3 µl EAM solution, loaded directly into the chip (direct spot). EAM alone was also loaded as a control. The chip was allowed to air dry for 15 min before being analysed.

IV.2.3. SELDI-TOF MS analysis

The chips with samples were placed into the vacuum chamber of the Ciphergen SELDI-TOF MS equipment and the crystals in each spot were hit with a laser, causing the proteins to desorb and ionize when the matrix absorbs the energy produced at the wavelength of the nitrogen laser. This results in ionized protein molecules in the gas phase. A brief electric field was then applied to accelerate the ions down a flight tube and a detector at the end of the tube recorded the time of flight. Given the time of flight, the known length of the tube and the voltage applied, the mass-to-charge ratio (*m*/*z* value) of the protein was derived. The typical spectrum obtained consisted of the sequentially recorded numbers of ions arriving at the detector (the intensity) coupled with the corresponding *m*/*z* value. The output from the system was then a full profile of proteins that bound to the chip and were detected. The mass spectrum contained peaks corresponding to proteins in the sample. The peak areas are proportional to the measured concentrations of the corresponding proteins. The proteins with altered expression (peaks with increased/decreased area) were selected and their molecular weight (MW) determined. To simplify, in this work peptides were considered to have MW < 7500 Da and proteins to have MW ≥ 7500 Da. Using the ExPASy software the putative proteins for a specified molecular weight and pI (the pI range was dependent on the pH of the buffer used) were tentatively identified.

IV.3. RESULTS

Mixtures of standard peptides and proteins were analysed. No protein peaks were visualised for EAM alone, as expected, and similar peaks were obtained in duplicates (Fig.IV.1). The molecular weights determined were used to calibrate the system.

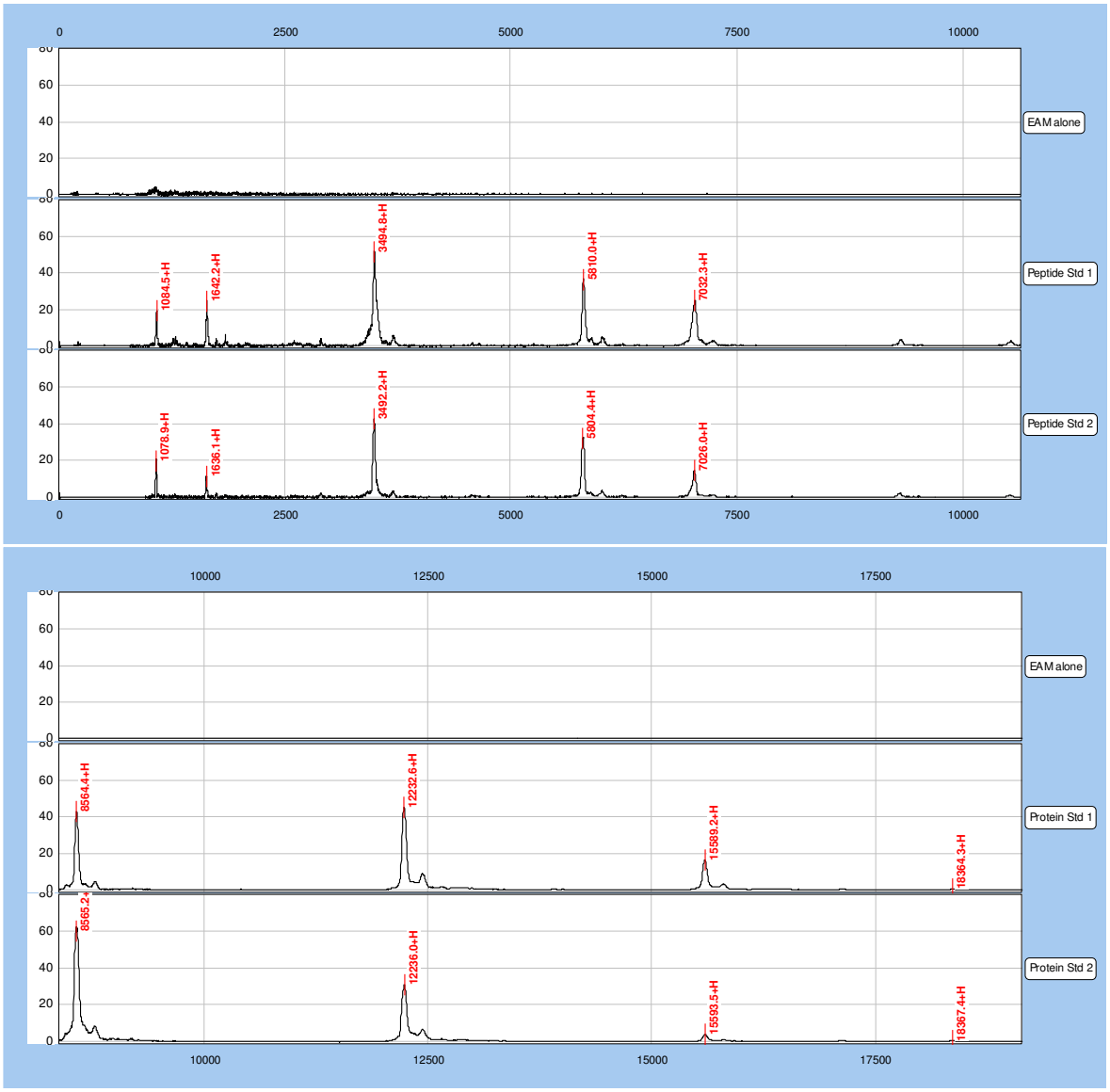

Fig. IV.1 - Peptide and protein standards SELDI-TOF MS spectra. Peptide standards, all-in-1 protein standards and EAM alone were loaded onto a NP20 chip by direct spotting and analysed. In the upper panel are presented the peptides obtained: Vasopressin - 1084.247 Da; Somatostatin - 1637.903 Da; Bovine insulin - 3495.941 Da; Human insulin - 5807.653 Da;, Hirudin - 7033.614 Da. In the lower panel are presented the proteins detected: Bovine Ubiquitin - 8564.8 Da; Bovine cytochrome c - 12230.9 Da; Bovine SOD - 15591.4 Da; Bovine β-Lactoglobulin - 18363.3 Da.

IV. 3.1. Aluminium-induced altered expression of proteins *in vitro*

The *in vitro* study was performed using PC12 and COS-1 cells. Quadruplicates of samples from PC12 or COS-1 cells incubated in medium "with serum", "without serum", in medium with 0.5 mM and 1 mM aluminium for 24 h or 48 h were loaded in different types of chips: H4 with PBS/25% ACN; NP20 with 50 mM HEPES (pH 7.4); SAX2 with 50 mM Tris (pH 9.0); SAX2 with 50 mM sodium acetate (NaOAc) (pH 4.5); WCX2 with 50 mM Tris (pH 9.0) and WCX2 with 50 mM NaOAc (pH 4.5). Full profiles of each were obtained. Proteins or peptides with altered expression are presented in detailed profiles in which the altered ones are labelled with the correspondent molecular weight.

For PC12 cells, no changes were observed in the expression of proteins detected with chips NP20 and SAX2 with NaOAc (pH 4.5). A full profile of the proteins detected using the SAX2 chip with Tris (pH 9.0) is presented on Fig. IV.2. In this profile a peptide of 6710.6 Da was detected exhibiting altered expression due to a serum effect, i. e., "with serum" samples revealed smaller peak areas than the others, for both 24 h and 48 h profiles (Fig. IV.3). Serum withdrawal apparently induced an increase in the expression of this peptide. Altered expression of 3250.2 Da, 3780 Da peptides and 9940.4 Da protein were detected by WCX2 with NaOAc (pH 4.5). The change in the expression was also apparently due to a serum effect. The 3250.2 Da and 3780 Da peptides (Fig. IV.4) revealed bigger peak areas for "with serum" samples, indicating a decrease in expression possibly due to serum withdrawal. A similar pattern of altered expression, albeit less marked, was also observed for a protein peak of 9940.4 Da (Fig. IV.5).

The expression of proteins in PC12 cells was also evaluated using a WCX2 chip and 50 mM Tris buffer (pH 9.0). A decrease of the expression of a protein with a molecular weight of 20245.5 Da due to aluminium was observed (Fig. IV.6). Samples from cells treated with 0.5 mM and 1 mM aluminium for 24 h and 48 h showed smaller peaks than the others. The putative protein found in the database corresponding to this molecular weight and to an expected pI>9.0 (dependent of the chip and pH buffer used) was the metalloproteinase inhibitor 1 precursor (MW: 20240.35, pI: 9.06). Although this was the strongest candidate, other hypothesis were the peptidyl-prolyl cis-trans isomerase B precursor (MW: 20218.15, pI: 9.42), the 60S ribosomal protein L11 (MW: 20152.21, pI: 9.64) and the microsomal signal peptidase 23 kDa subunit (MW: 20146.43, pI: 9.61).

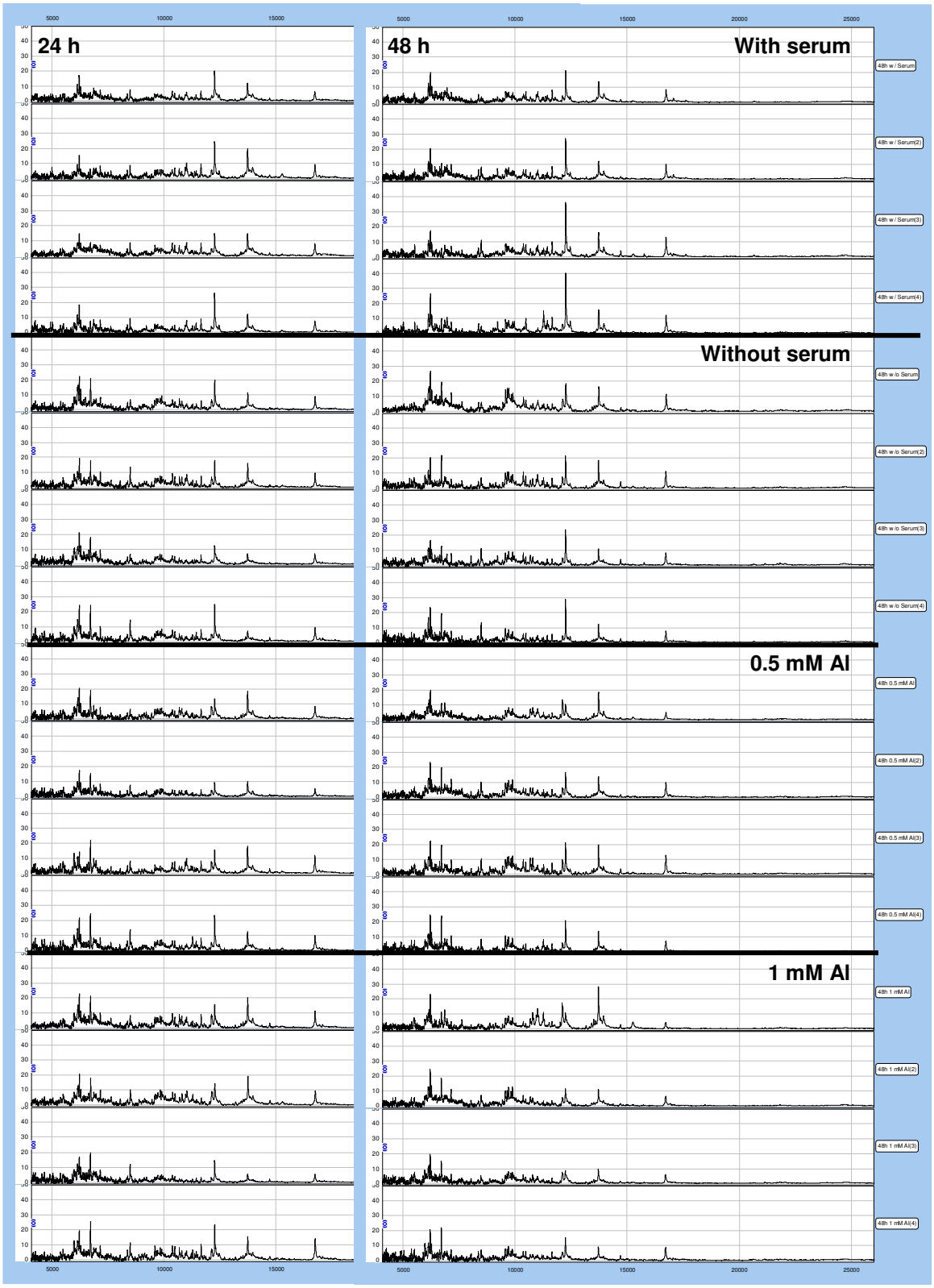

Fig. IV.2 – Examples of SELDI-TOF MS spectra. The full profiles of proteins (peaks) were detected using SAX2, 50 mM Tris buffer (pH 9.0). Left profiles are from 24 h samples and right ones from 48 h samples. The upper group of 4 spectra are from quadruplicates of "with serum" samples, the other groups of 4 spectra are from quadruplicates of "without serum", "0.5 mM Al" and "1 mM Al" samples, respectively. The *Y*-axis is a relative intensity scale and the *X*-axis is a mass-to-charge ratio (*m*/*z*) scale (Da).

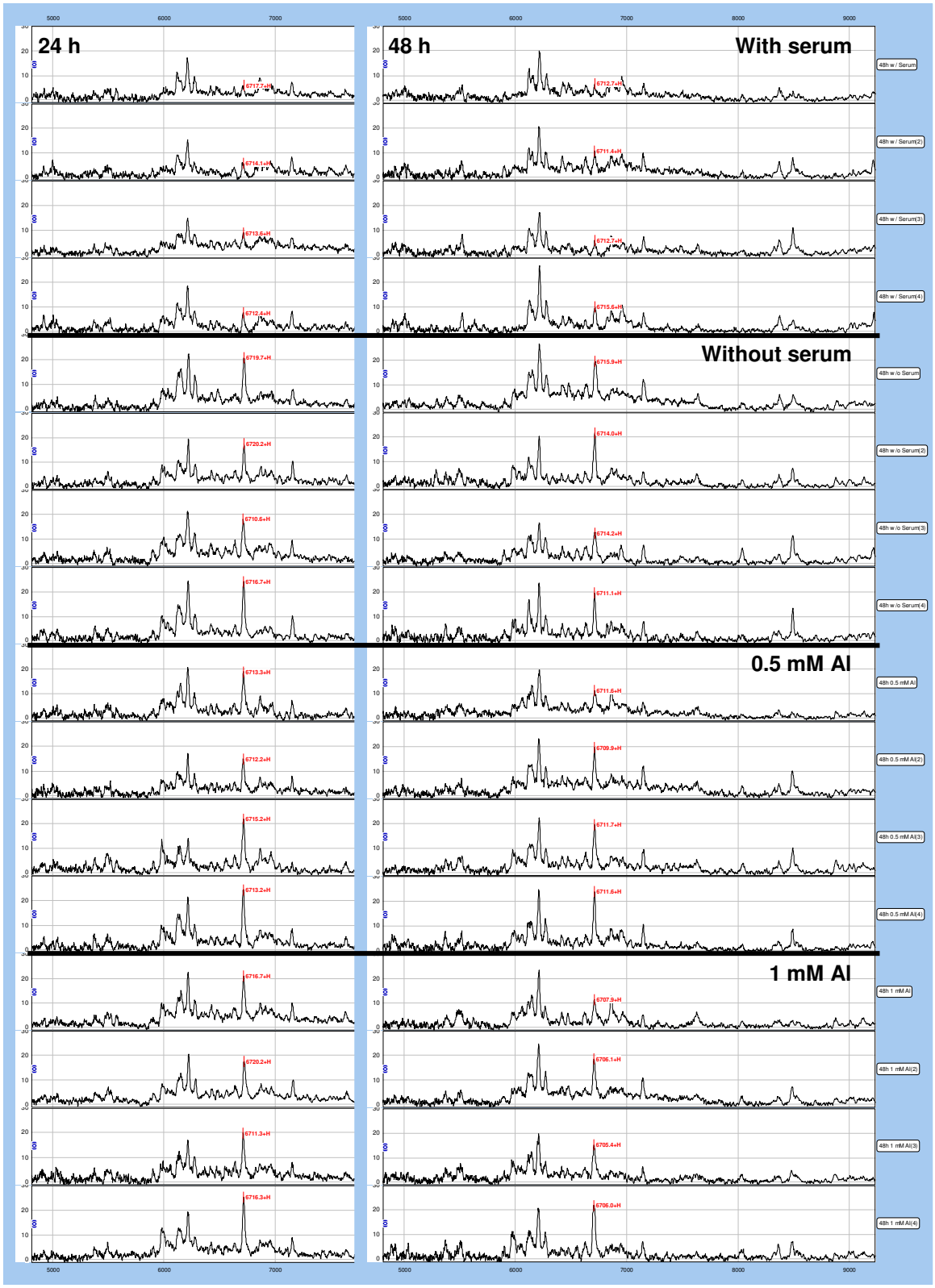

Fig. IV.3 - Altered expression of 6710.6 Da peptide due to serum withdrawal in PC12. The peptide was detected using SAX2, 50 mM Tris buffer (pH 9.0). Altered peaks are labelled with the corresponding calculated molecular weight.

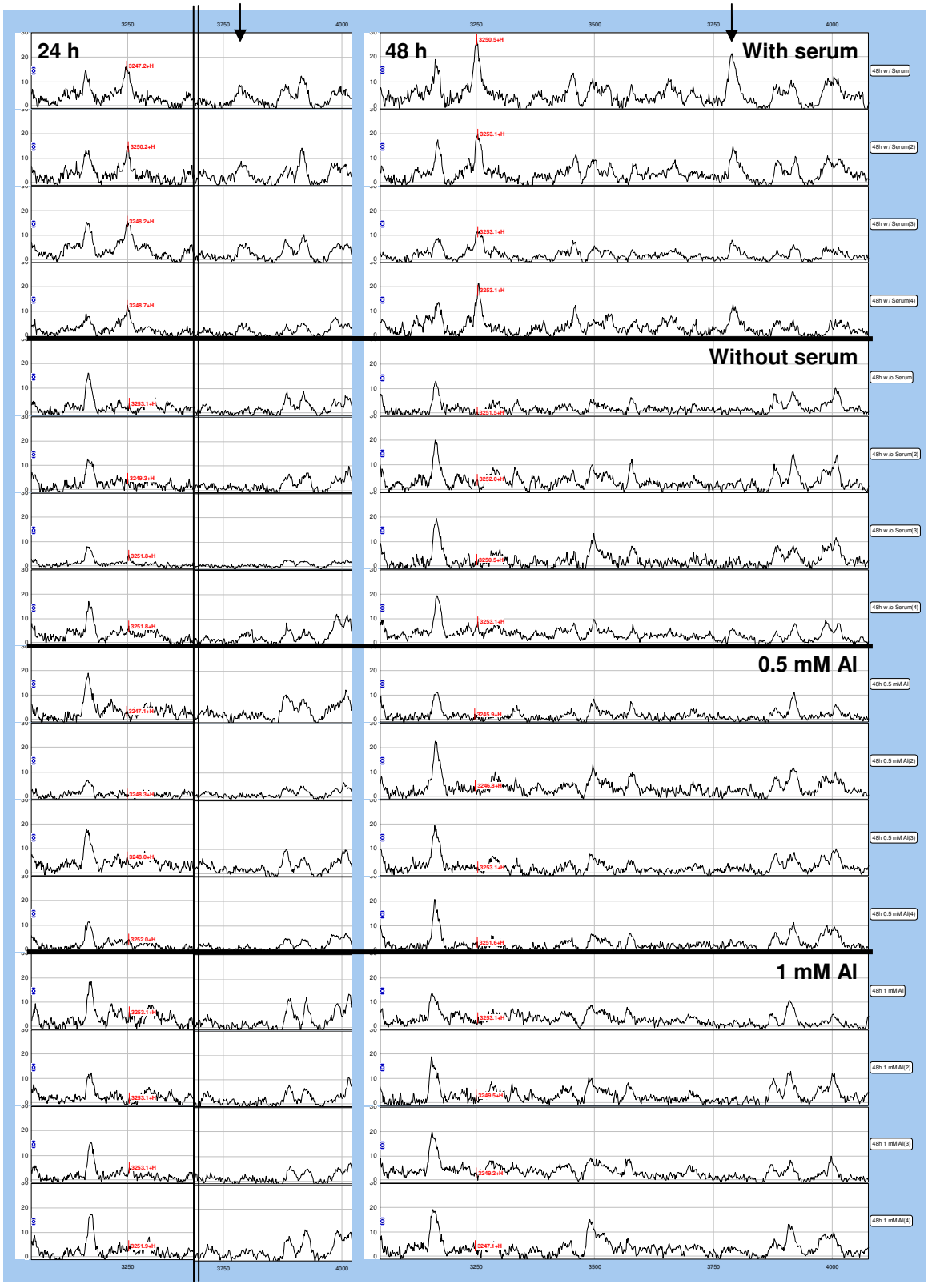

Fig. IV.4 – Serum withdrawal induced decreased expression of 3250.2 Da and 3780 Da peptides in PC12 cells. The peptides were detected using WCX2, 50 mM NaOAc buffer (pH 4.5). The 24 h spectra were cut to better show both altered peptides, as indicated by the vertical double lines.

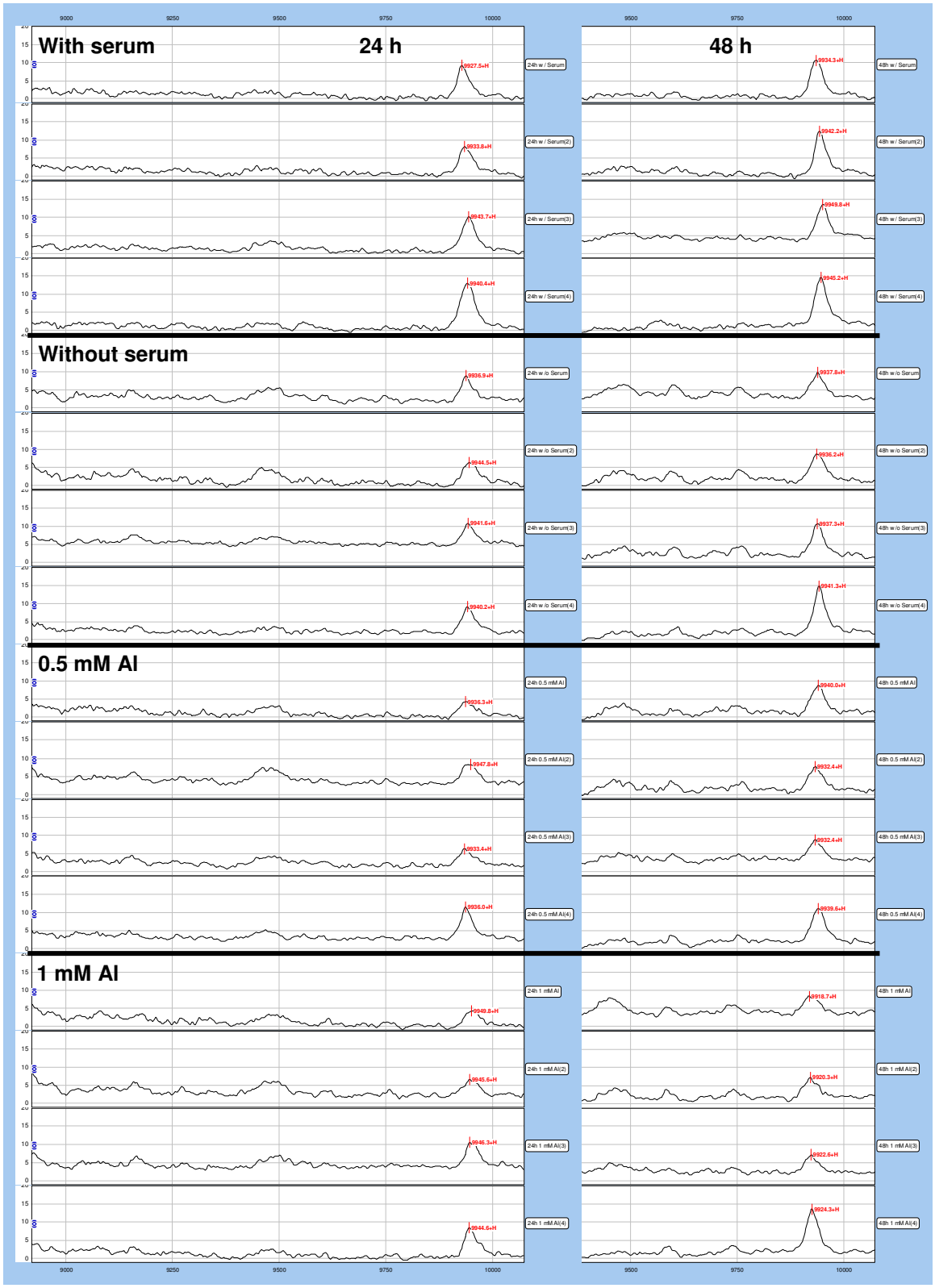

Fig. IV.5 - Altered expression of a 9940.4 Da protein due to serum withdrawal in PC12 cells. The protein was detected using WCX2, 50 mM NaOAc buffer (pH 4.5).

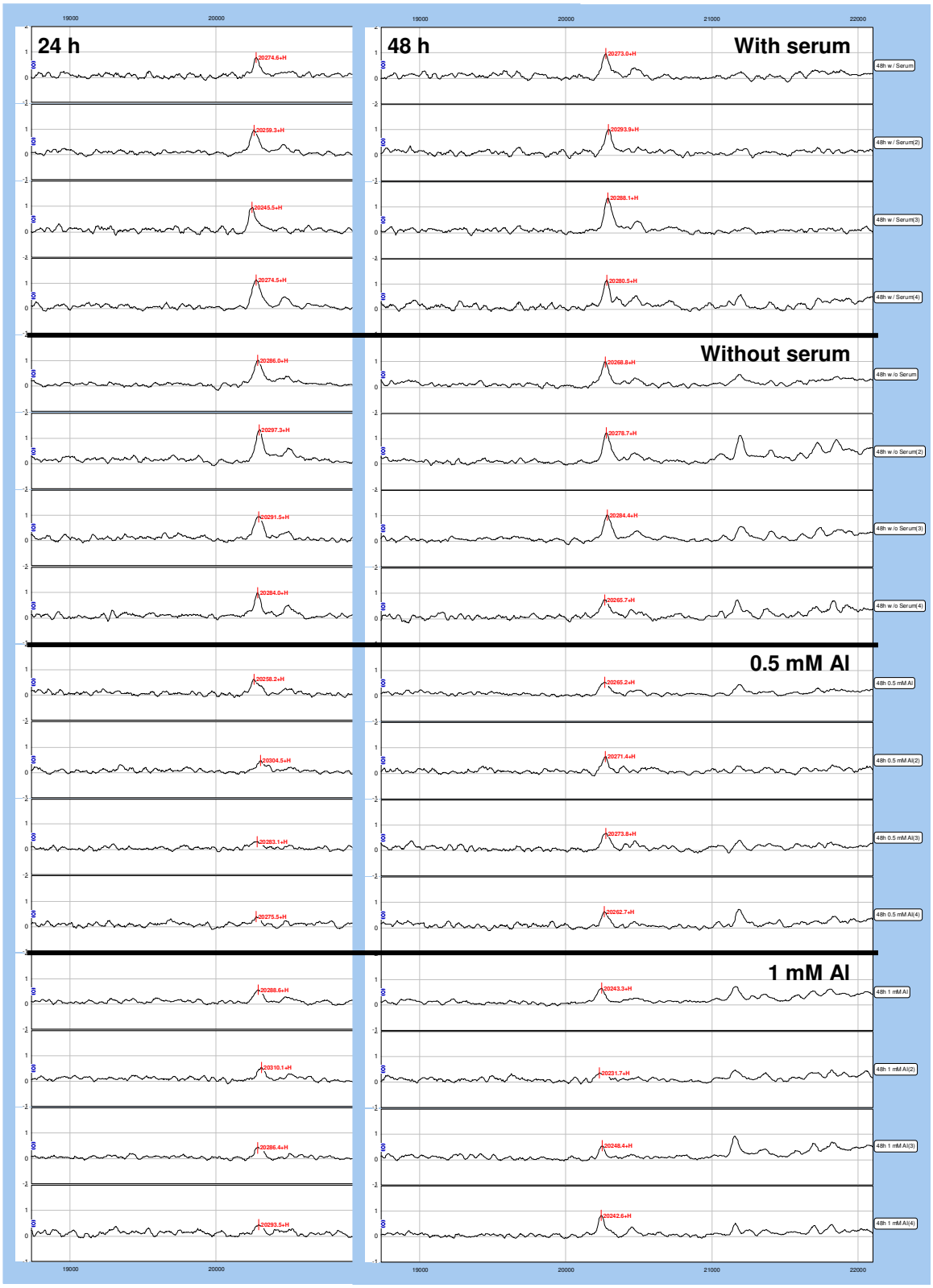

Fig. IV.6 - Aluminium induced change in protein expression in PC12 cells. A protein with 20245.5 Da was detected using a chip WCX2 and Tris (pH 9.0).

In COS-1 cells full profiles of proteins were also detected using different types of chips: H4 with PBS/25% ACN; NP20 with 50 mM HEPES (pH 7.4); SAX2 with 50 mM Tris (pH 9.0); SAX2 with 50 mM NaOAc (pH 4.5); WCX2 with 50 mM Tris (pH 9.0) and WCX2 with 50 mM NaOAc (pH 4.5). No alterations in protein expression were detected on proteins captured in the chips NP20, SAX2 with 50 mM Tris (pH 9.0) and WCX2 with 50 mM Tris (pH 9.0).

A few proteins were detected whose expression altered apparently due to serum withdrawal. The proteins with 7840.6 Da and with 8200.7 Da detected with the chip SAX2 with 50 mM NaOAc (pH 4.5) revealed decreases in their expression, but only in the 48 h profile (Fig. IV.7). Another peak, not identified in the figure, with a molecular weight of 7967.1 Da was also detected exhibiting the same expression pattern of the others.

The chip WCX2 with 50 mM NaOAc (pH 4.5) allowed the detection of two peptides with altered expression. The peptides with 4377.8 Da and 4776.4 Da showed a double effect in their expression, a first decrease due to serum withdrawal and an extra decrease due to aluminium exposure. Those effects were only verified in the 48 h profiles, as presented in Fig. IV.8A and Fig. IV.8B, respectively.

Two proteins were detected with altered expression due to aluminium exposure. A protein of 9501.4 Da was discovered using a chip WCX2 with 50 mM NaOAc (pH 4.5). Its expression exhibited a decrease induced by aluminium, in both 24 h and 48 h profiles (Fig. IV.9). The search in the database for putative proteins revealed the most probable to be Stannin (MW: 9497.10, pI: 5.19). Other less likely candidates were Glycoprotein J (MW: 9510.33, pI: 9.13) and the ATPase inhibitor, mitochondrial precursor (MW: 9516.56, pI: 7.21).

The other, was a protein of 10825.4 Da detected using a H4 chip with PBS/25% ACN buffer. An increase in its expression was verified in samples treated with aluminium (Fig. IV.10). However, this effect was transient, since it was only verified in the 24 h profile. The putative protein found in the database as being the most probable was the genome polyprotein (MW: 10823.66, pI: 5.28). Other less likely possibilities include the leukocyte specific transcript 1 protein (MW: 10822.42, pI: 8.43), the polyprotein (MW: 10819.82, pI: 8.83), the E7 protein (MW: 10819.14, pI: 4.02), Calgranulin A (MW: 10834.51, pI: 6.51) and the E7 protein (MW: 10837.13, pI: 4.33).

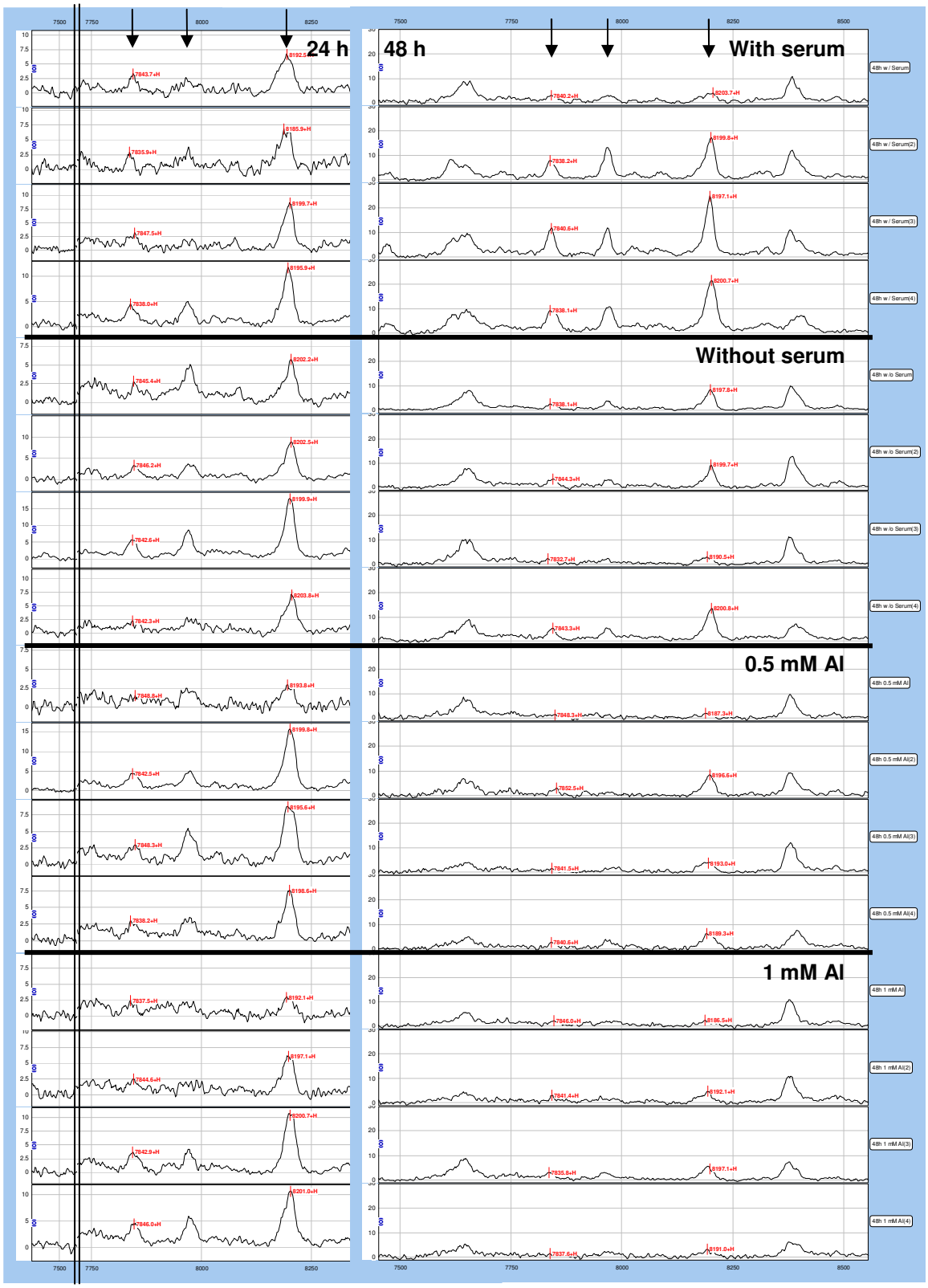

Fig. IV.7 – Detection of altered protein expression in COS-1 cells. The proteins with 7840.6 Da, 7967.1 Da and with 8200.7 Da were detected using the chip SAX2 with 50 mM NaOAc (pH 4.5). Decreases in their expression were only verified in the 48 h profiles. The 24 h panel was cut to better show altered proteins, as indicated by the double vertical lines.

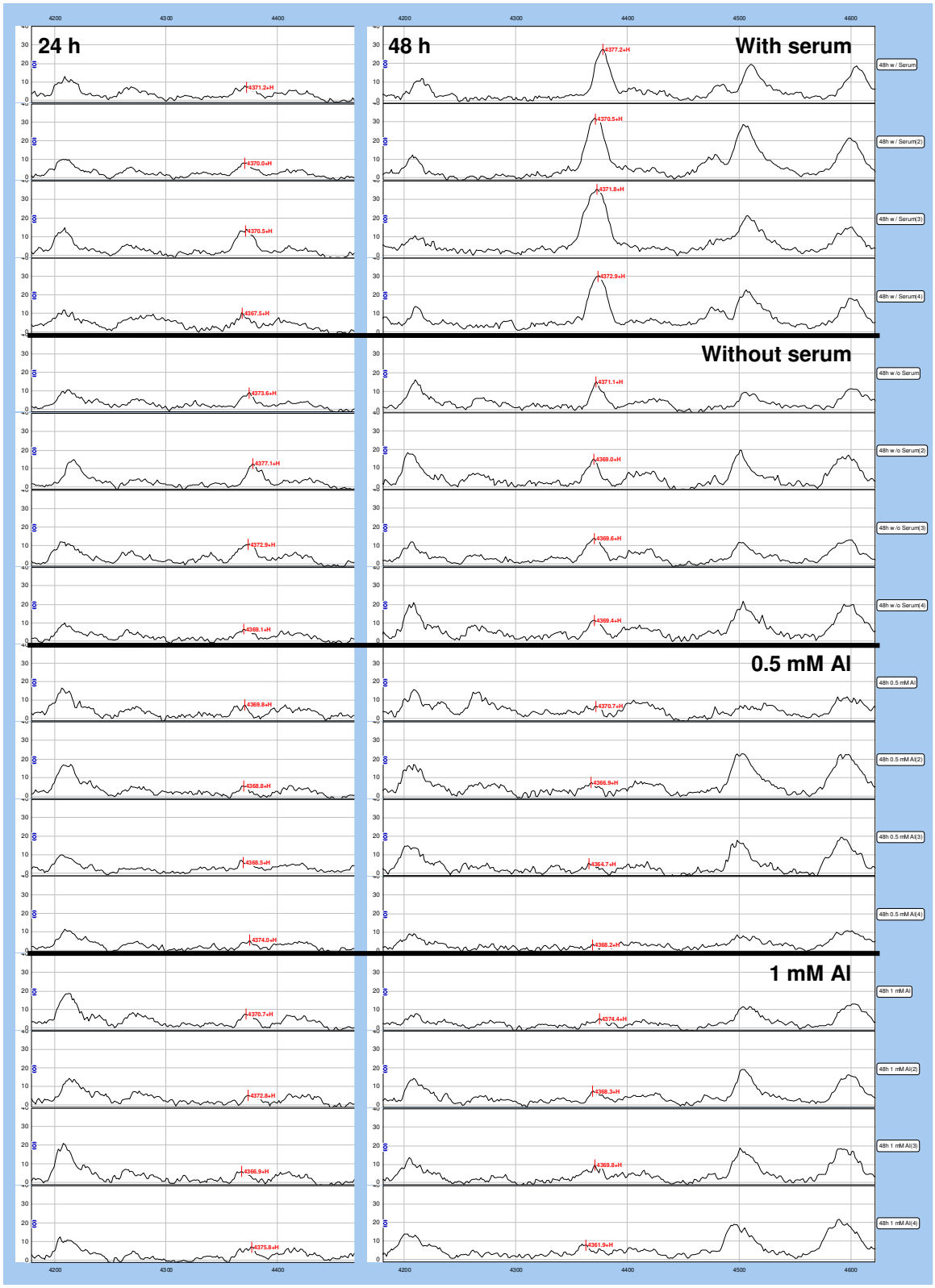

Fig. IV.8A – Altered peptide expression in COS-1 cells. The expression of a peptide of 4377.8 Da showed a double effect strongly evident in the 48 h panel. The chip used was WCX2 and 50 mM NaOAc (pH 4.5).

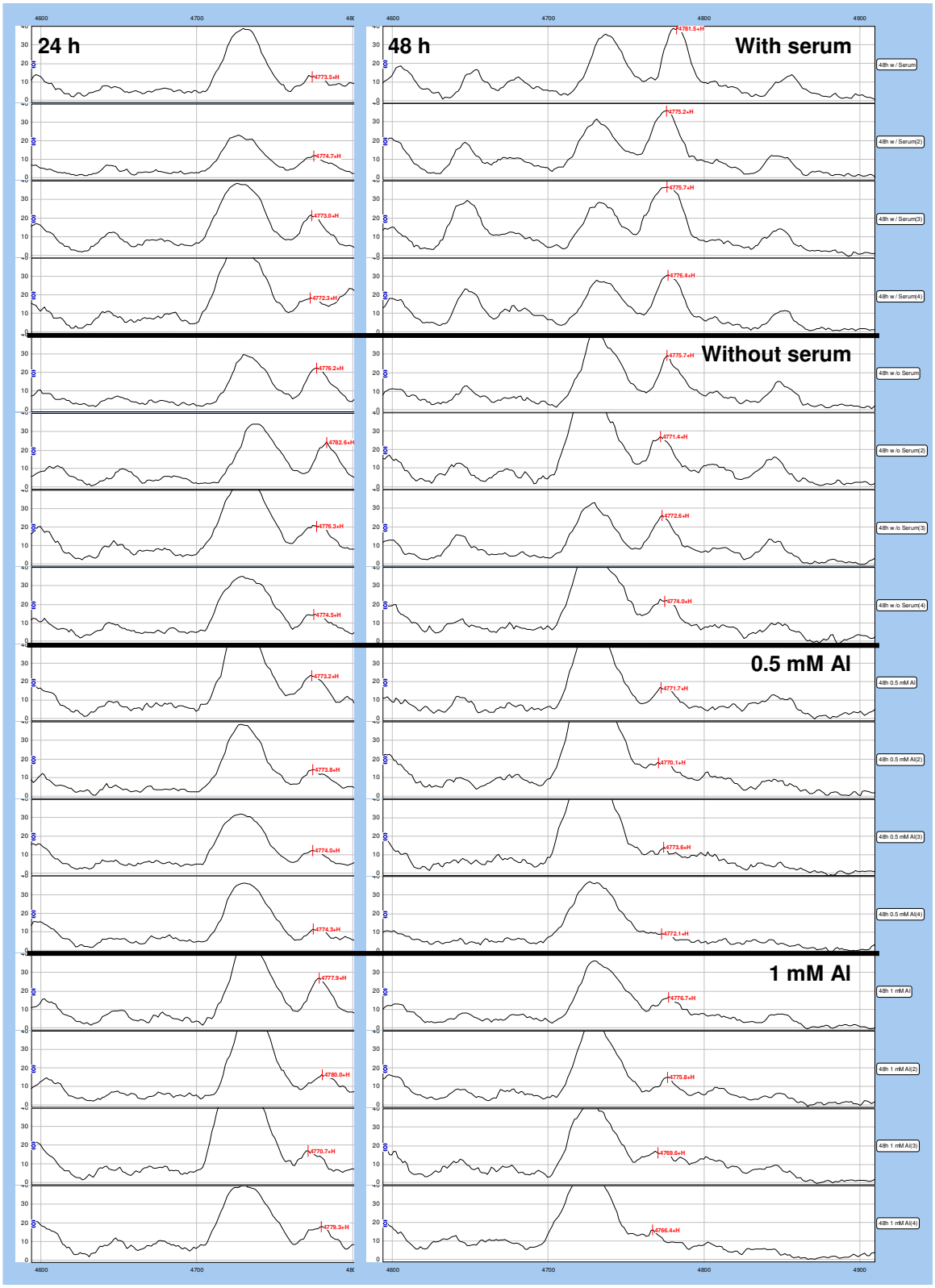

Fig. IV.8B – Altered peptide expression in COS-1 cells. A peptide of 4776.4 Da was detected using the chip WCX2 and 50 mM NaOAc (pH 4.5) whose altered expression is more evident in the 48 h profile.

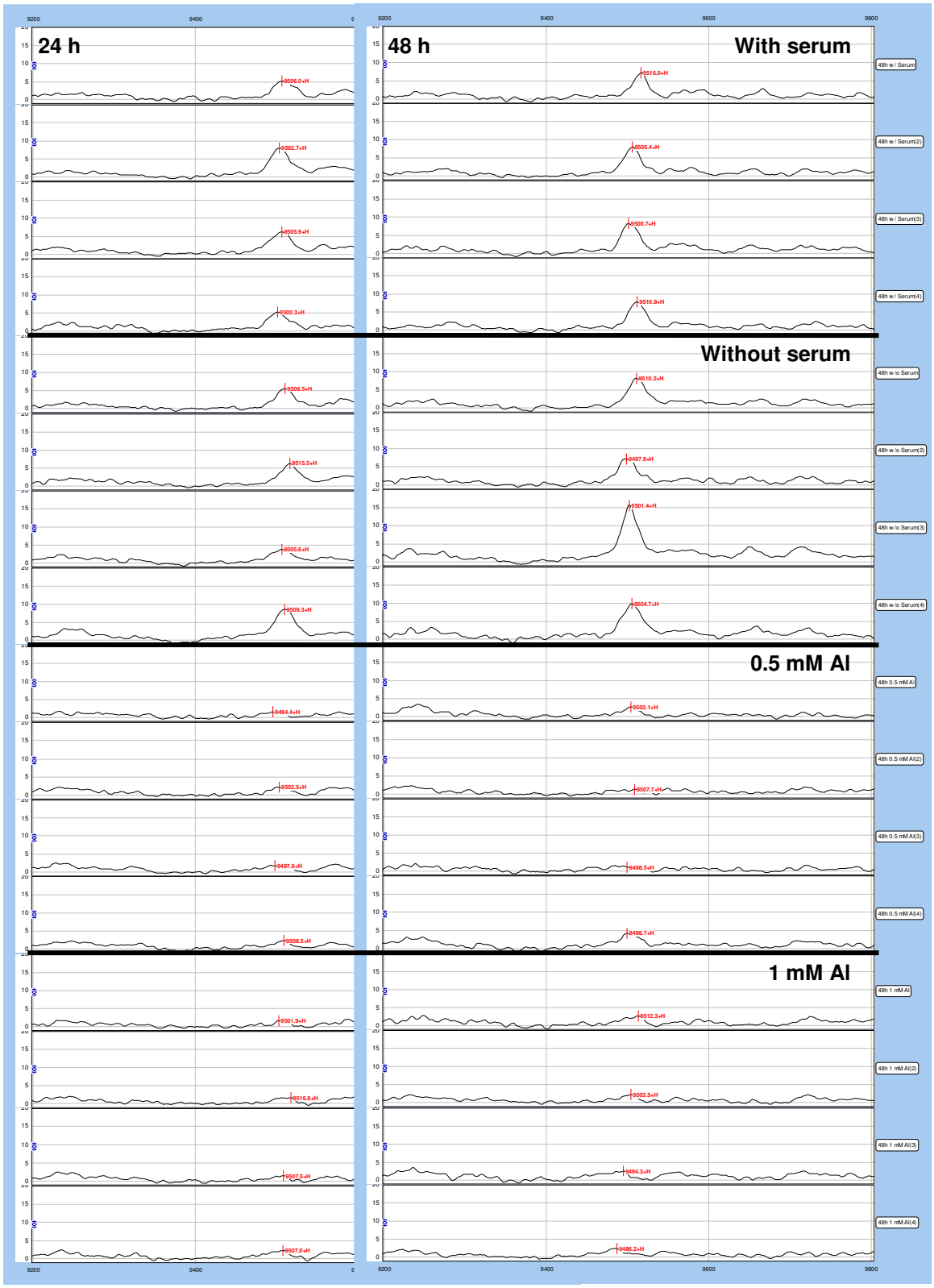

Fig. IV.9 - Aluminium induced altered expression of a 9501.4 Da protein in COS-1 cells. The protein was identified using a WCX2 chip with 50 mM NaOAc (pH 4.5). The decrease in the expression induced by aluminium may be observed in both 24 h and 48 h profiles.

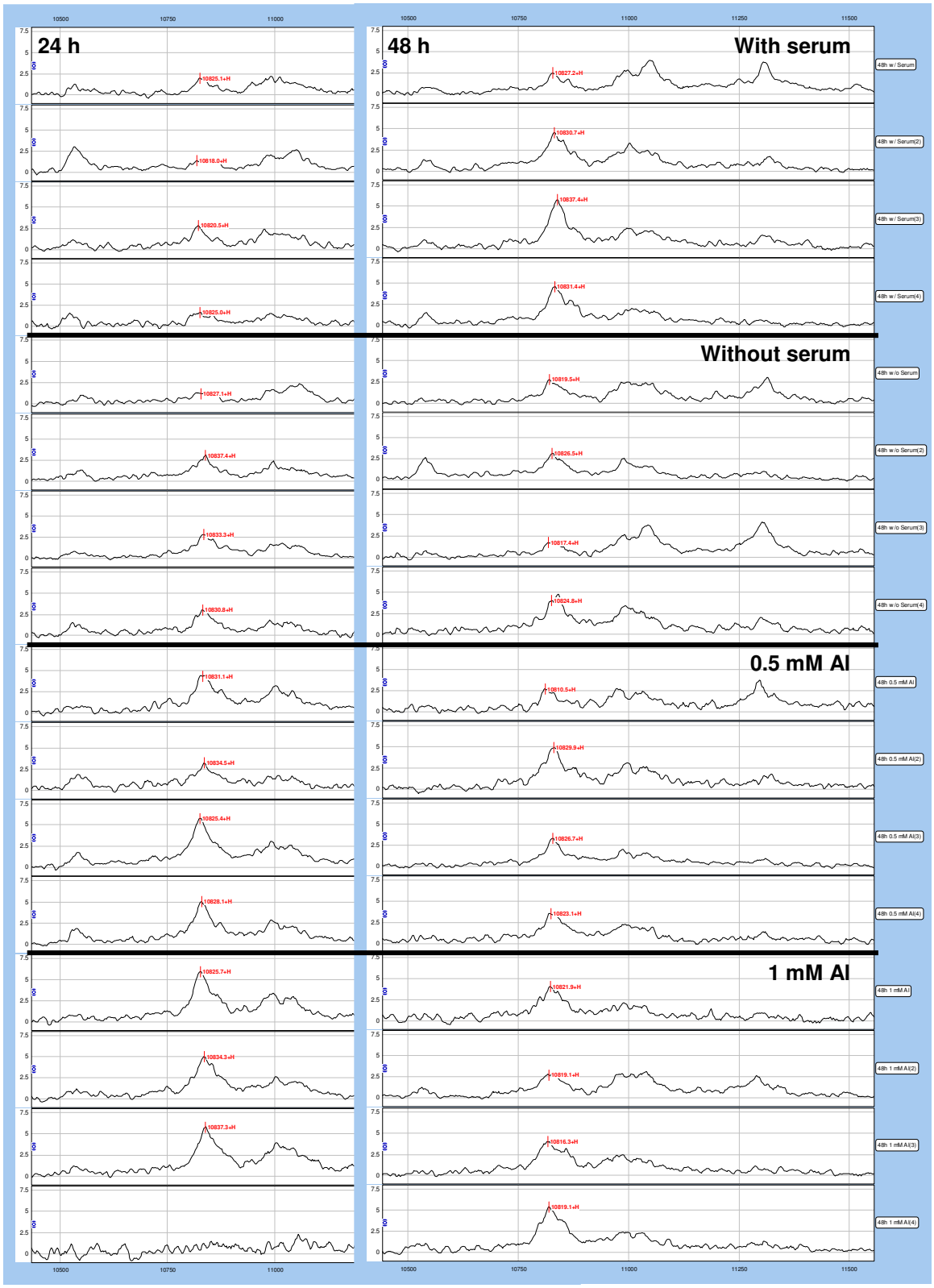

Fig. IV.10 – Detection of a protein with altered expression due to aluminium in COS-1 cells. The protein of 10825.4 Da was detected using a H4 chip with PBS/25% ACN buffer. The increase in the expression was only verified in the 24 h profile.

IV. 3.2. Aluminium-induced altered expression of proteins *in vivo*

For the *in vivo* study, mice were injected with aluminium lactate and sacrificed after 3 h or 24 h. Duplicates of "sham" and triplicates of "saline" and "aluminium" samples were loaded in different matrix chips and treated with different buffers: H4 with PBS/0.5 M NaCl; IMAC3 with PBS/0.5 M NaCl; NP20 with 50 mM HEPES (pH 7.4); SAX2 with 50 mM Tris (pH 9.0); SAX2 with 50 mM NaOAc (pH 4.5); WCX2 with 50 mM Tris (pH 9.0) and WCX2 with 50 mM NaOAc (pH 4.5). Full profiles of each were obtained.

No changes were observed in the expression of proteins detected by the chips SAX2 treated with 50 mM Tris (pH 9.0) or with 50 mM NaOAc (pH 4.5) and by WCX2 with 50 mM Tris (pH 9.0). However, with the chip H4 and PBS/0.5 M NaCl, a protein of 9805.9 Da was discovered which showed increased expression due to aluminium, but only in animals treated for 24 h (Fig. IV.11).

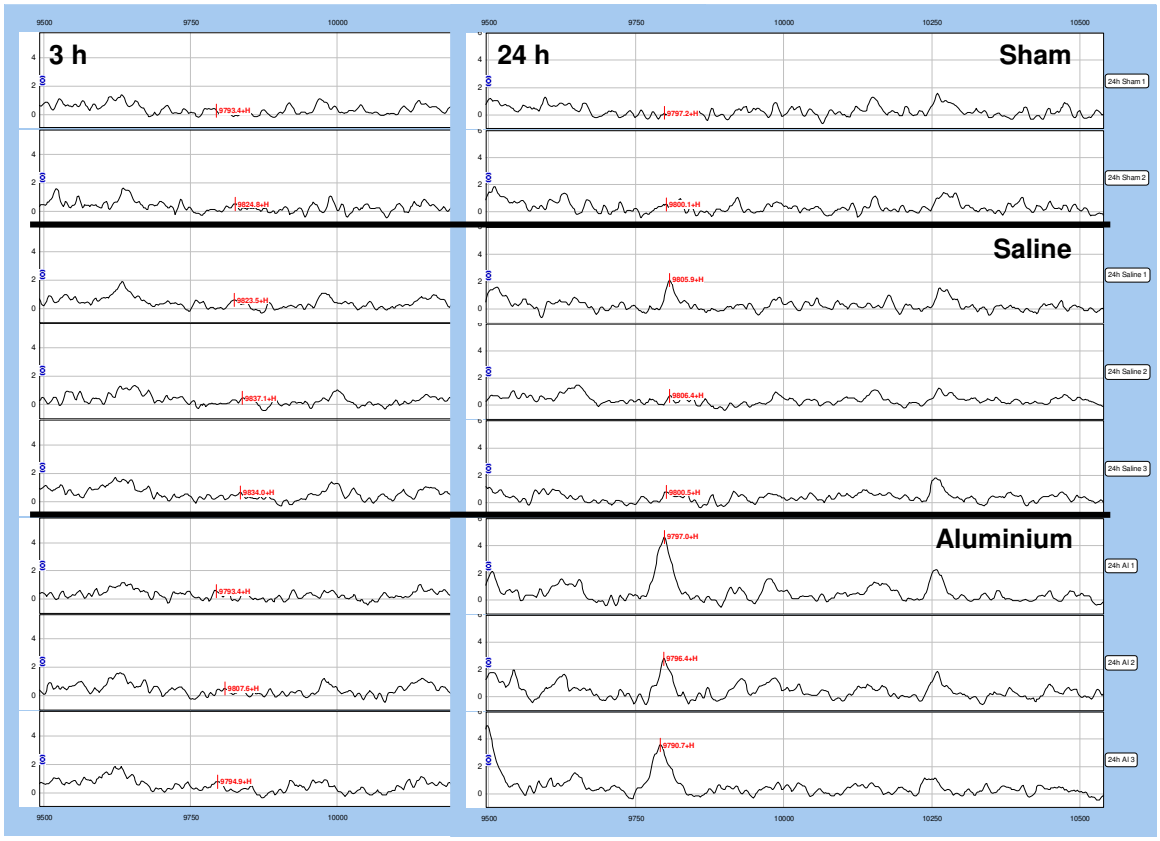

Fig. IV.11 - Increased expression of a 9805.9 Da protein in mice after 24 h aluminium treatment. The protein was detected using a H4 chip with PBS/0.5 M NaCl buffer. Profiles are from 3 h samples (left) and from 24 h samples (right). The upper group of 2 spectra are duplicates of "sham" samples, the other groups of 3 spectra are from triplicates of "saline" and "aluminium" samples, respectively. The *Y*-axis is a relative intensity scale and the *X*-axis is a mass-to-charge ratio (*m*/*z*) scale (Da). Altered peaks are labelled with the correspondent molecular weight.

The search for putative proteins in the database revealed the main candidate to be the U6 snRNA-associated Sm-like protein LSm5 (MW: 9806.3 Da, pI: 4.42). Other putative candidates were the Anaphase promoting complex subunit 11 (MW: 9817.56 Da, pI: 7.99) and the Small inducible cytokine B13 precursor (MW: 9790.65 Da, pI: 10.52).

The chip IMAC3 (with PBS/0.5 M NaCl) was only used to analyse the 24 h samples. A peptide of 6160.8 Da was detected with decreased expression in the aluminium treated samples. A 7660.2 Da protein was also found exhibiting decreased expression due to aluminium. Both profiles are presented in Figure IV.12.

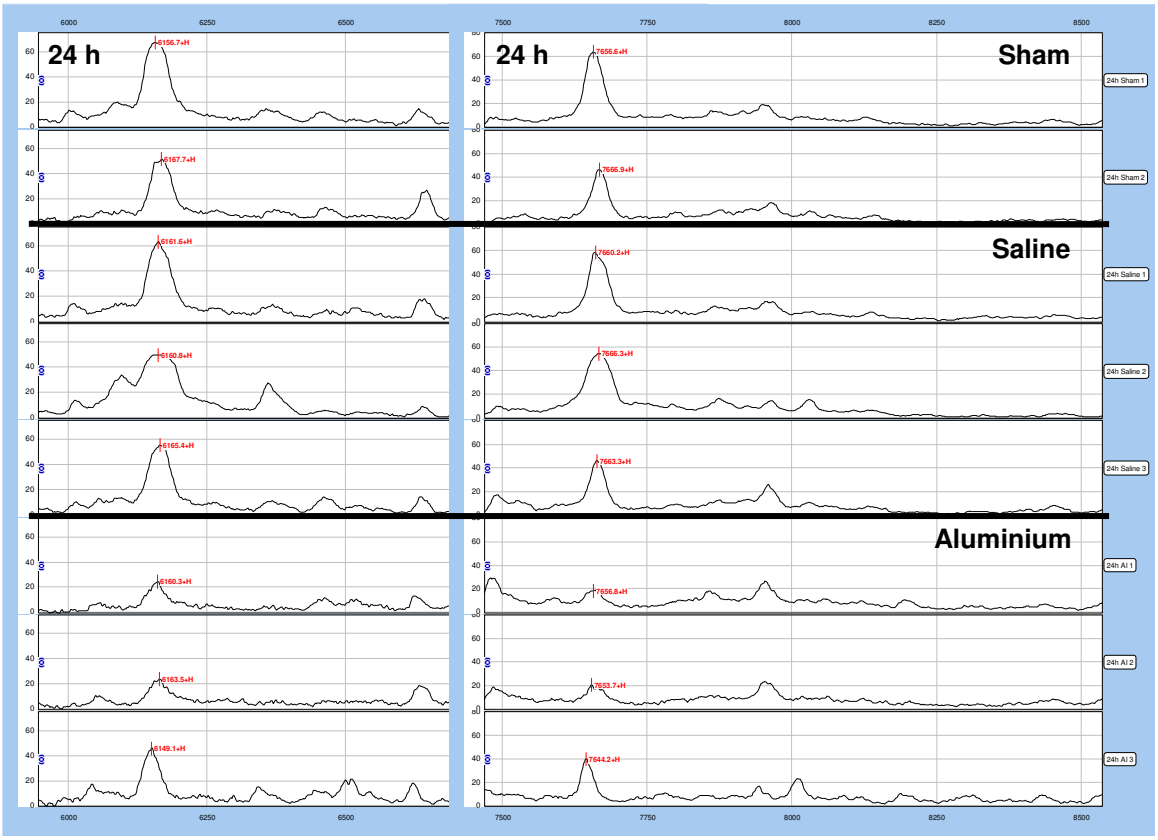

Fig. IV.12 - Aluminium-induced altered expression of a peptide and of a protein. The peptide with 6160.8 Da and the protein with 7660.2 Da were detected with the chip IMAC3 with PBS/0.5 M NaCl. Only the 24 h profiles are shown.

The putative proteins identified from the data search for the 6160.8 Da peptide were the Beta-defensin 11 precursor (MW: 6165.25 Da, pI: 8.31), the Serine protease inhibitor Kazal-type 3 precursor (MW: 6126.08 Da, pI: 7.8) and the Metallothionein-2 (MW: 6115.22 Da, pI: 8.23). For the 7660.2 Da protein, the putative proteins identified from the

database were the Guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-13 subunit (MW: 7651.95 Da, pI: 5.38), the Insulin-like growth factor I precursor (Somatomedin) (MW: 7676.88 Da, pI: 8.31) and the DNA-directed RNA polymerase II 7.6 kDa polypeptide (MW: 7645.12 Da, pI: 7.65).

Two peptides were detected with altered expression using the chip NP20 with 50 mM Hepes (pH 7.4). A peptide of 6556.9 Da exhibited a decrease in its expression in aluminium treated samples. This effect was more obvious on the 3 h profiles than on the 24 h profiles (Fig. IV.13). The expression of a 6896.6 Da peptide also decreased in aluminium treated samples but, only on the 24 h profiles, although in relation to the 3 h profiles, in the 24 h profiles an increase in the expression of this peptide was verified on sham and saline samples, indicating a saline effect.

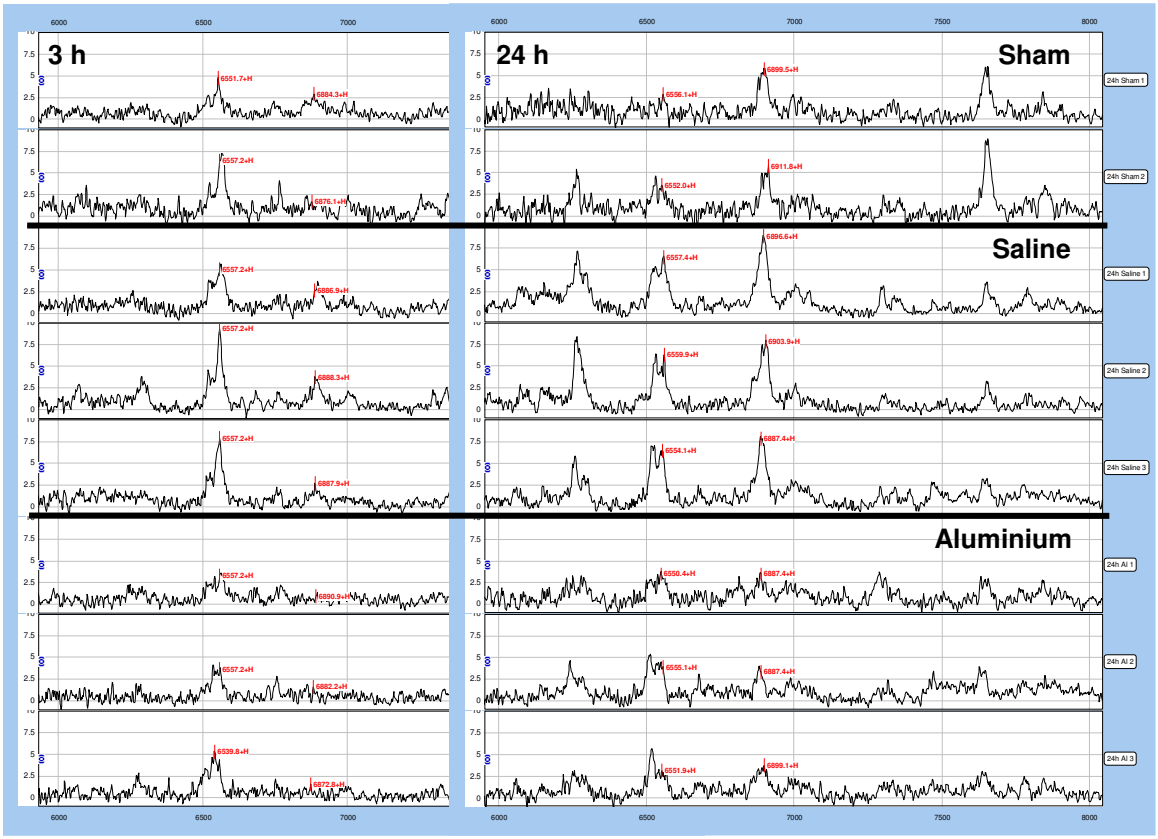

Fig. IV.13 – Detection of two peptides with altered expression in mice. The peptides of 6556.9 Da and 6896.6 Da were detected using the chip NP20 with 50 mM Hepes (pH 7.4).

Putative proteins identified for the 6556.9 Da peptide were the 40S ribosomal protein S29 (MW: 6545.57 Da, pI: 10.17), the Ubiquinol-cytochrome c reductase complex

6.4 kDa protein (MW: 6538.64 Da, pI: 9.81), the Beta-defensin 29 precursor (MW: 6541.71 Da, pI: 9.5) and the Cytochrome c oxidase polypeptide VIIa-liver/heart, mitochondrial precursor (MW: 6572.66 Da, pI: 9.11). For the 6896.6 Da peptide the Small EDRK-rich factor 2 (4F5rel) (MW: 6899.85 Da, pI: 10.44) and the APP gamma-secretase C-terminal fragment 59 (MW: 6834.89 Da, pI: 7.03) were identified as possible candidates.

Also, with the chip NP20 and with 50 mM Hepes (pH 7.4), a protein of 13821.5 Da was detected with altered expression. The expression decreased due to aluminium on both animal groups (3 h and 24 h), with the effect being more pronounced in the 24 h profile (Fig. IV.14).

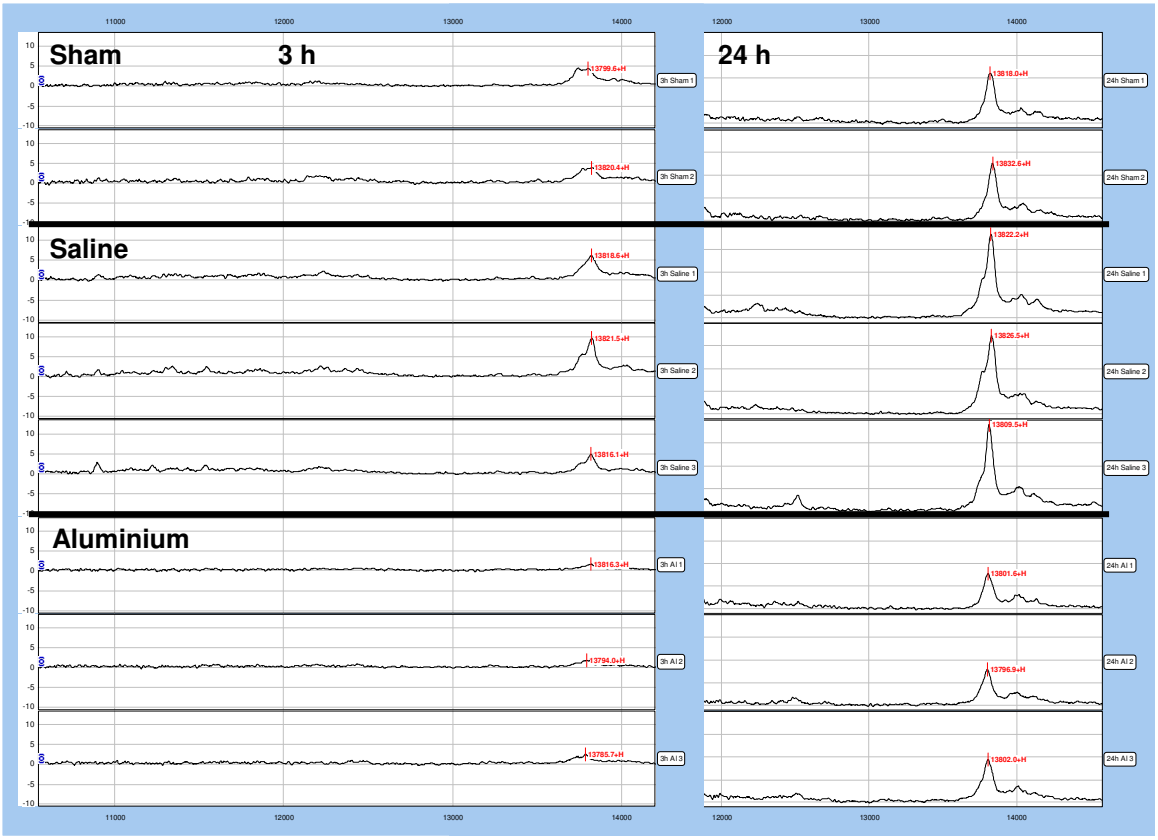

Fig. IV.14 – Detection of a 13821.5 Da protein exhibiting aluminium induced altered expression. The protein was detected using the chip NP20 with 50 mM Hepes (pH 7.4).

From the search in the database for putative proteins, the Peptidyl-propyl cis-trans isomerase NIMA-interacting 4 (MW: 13814.91 Da, pI: 9.78) was the best candidate. Other probable proteins found were the Tumor necrosis factor receptor superfamily member 23 precursor (MW: 13831.54 Da, pI: 5.04), the Calcium-dependent phospholipase A2

precursor (MW: 13802.83 Da, pI: 8.25), and the Small inducible cytokine A2 (MW: 13847.89 Da, pI: 9.81).

Using the chip WCX2 with 50 mM NaOAc (pH 4.5) two peptides of 4845.3 Da and 5232.4 Da were detected exhibiting a decrease in their expression due to aluminium, but only in the 24 h profiles (Fig. IV.15).

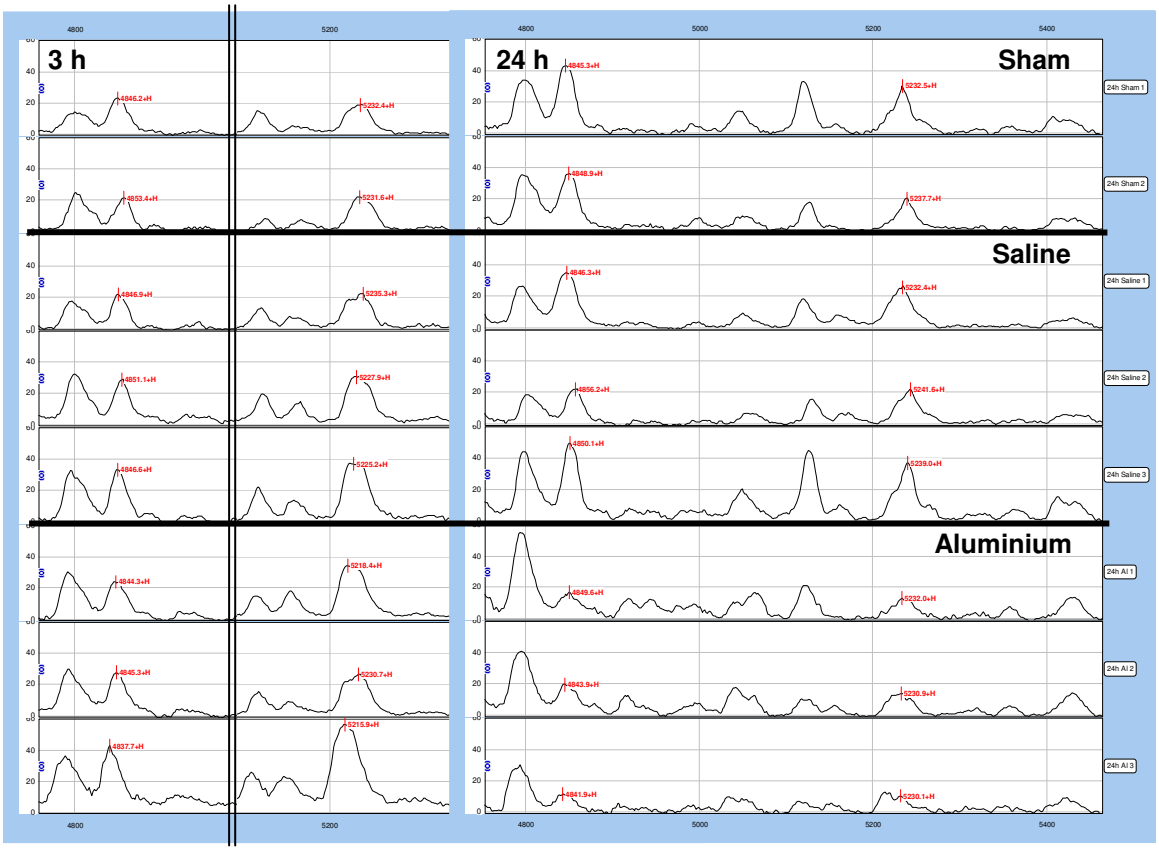

Fig. IV.15 – Detection of peptides with altered expression due to aluminium in mice. Peptides of 4845.3 Da and 5232.4 Da were detected using the chip WCX2 with 50 mM NaOAc (pH 4.5). The 3 h panel was cut to better show both peptides, as indicated by the double vertical lines.

The database search for putative proteins for the peptide with 4845.3 Da identified the Cathepsin H light chain (MW: 4821.57 Da, pI: 8.01), the Thymosin beta-10 (MW: 4894.48 Da, pI: 5.32) and the Cytochrome c oxidase polypeptide VIII-liver, mitochondrial precursor (MW: 4898.78 Da, pI: 7.93) as the best candidates. For the 5232.4 Da peptide, the Cocaine-and amphetamine-regulated transcript protein CART(55-102) (MW: 5265.26 Da, pI: 8.87) and Adrenomedullin-2 (MW: 5203.94 Da, pI: 9.71) were considered the better candidates.

With the same chip WCX2 and also with 50 mM NaOAc (pH 4.5), two other proteins were also detected with altered expression (Fig. IV.16). The expression of a protein with 7103.1 Da slightly increased due to aluminium at 3 h and more evidently at 24 h. The expression of a protein with 7219.2 Da also increased due to aluminium, but only at 24 h.

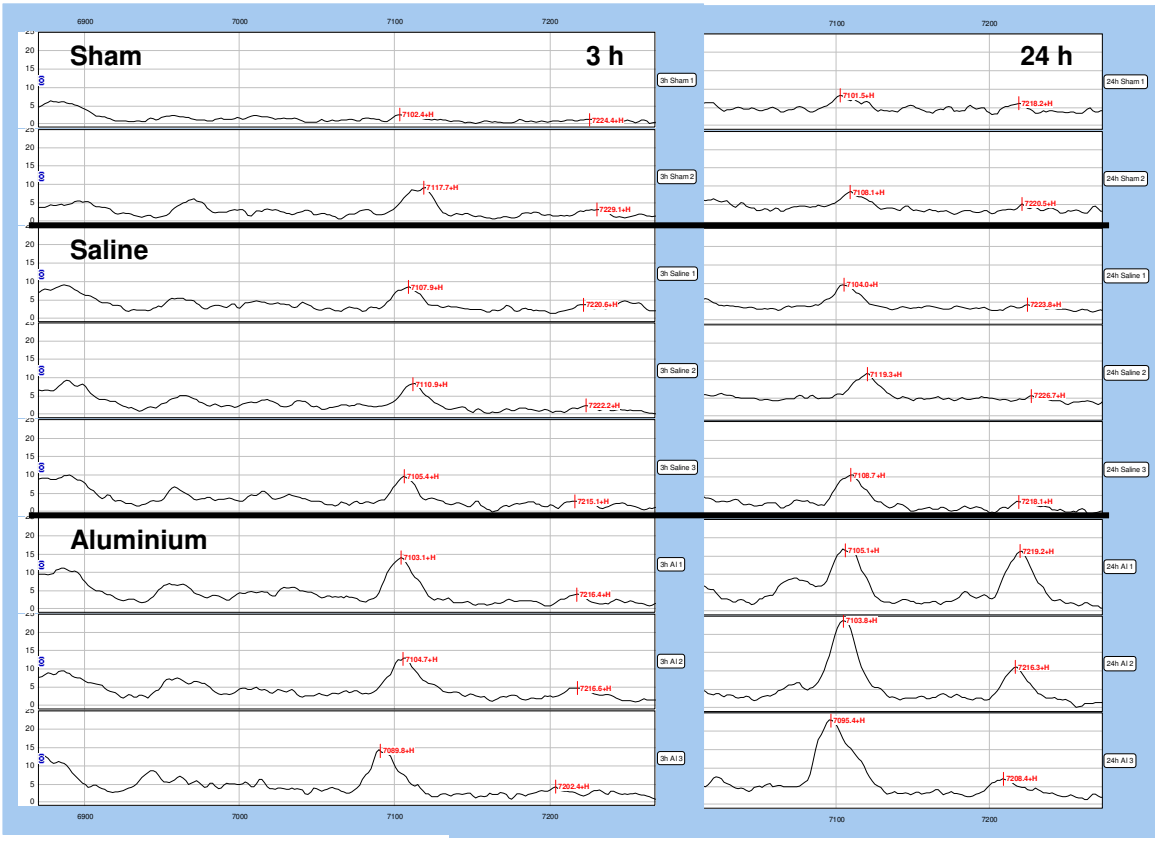

Fig. IV.16 – Detection of two proteins with aluminium-induced increased expression. The mouse proteins of 7103.1 Da and 7219.2 Da were detected using the chip WCX2 with 50 mM NaOAc (pH 4.5).

The putative proteins identified from the database search for the 7103.1 Da protein was the Guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-7 subunit (G-protein) (MW: 7140.2 Da, pI: 8.71), and for the 7219.2 Da protein was the FXYD domain-containing ion transport regulator 4 precursor (Channel inducing factor) (MW: 7168.35 Da, pI: 8.43).

Two more proteins, of 11168.1 Da and 11644.5 Da, were also noticed exhibiting aluminium-induced increased expression after 24 h, using the same chip [WCX2 with 50 mM NaOAc (pH 4.5)] (Fig. IV.17).

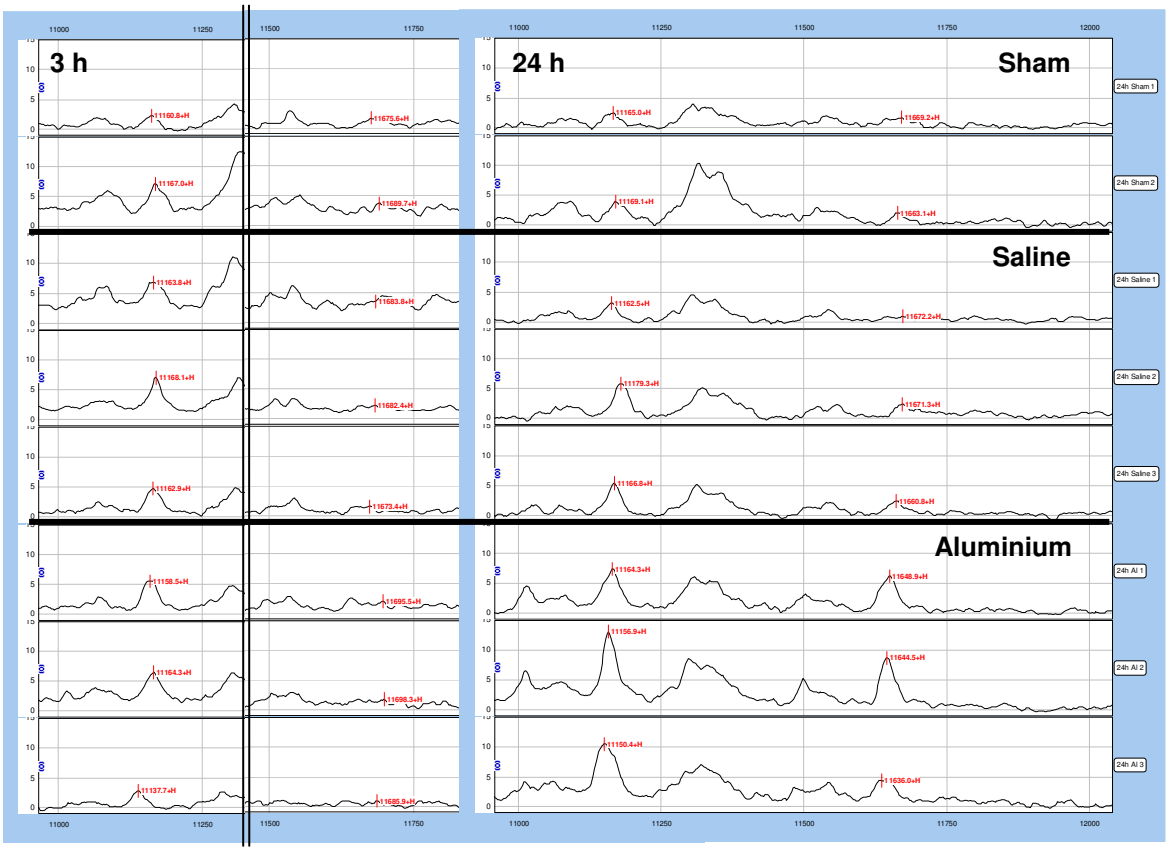

Fig. IV.17 – Detection of increased expression of two proteins induced by aluminium in mice. The proteins of 11168.1 Da and 11644.5 Da were detected using the chip WCX2 with 50 mM NaOAc (pH 4.5). The 3 h panel was cut to better show both altered proteins, as indicated by the double vertical lines.

The putative proteins identified from the database search for the 11168.1 Da protein were the Ig heavy chain V region 1B43 precursor (MW: 11168.41 Da, pI: 7.97), the Ig heavy chain V region M315 precursor (MW: 11167.42 Da, pI: 8.6), the S100 calcium-binding protein A13 (MW: 11157.8 Da, pI: 5.89) and the Death-associated protein 1 (MW: 11154.53 Da, pI: 9.35). For the 11644.5 Da protein the putative proteins identified were the Transmembrane protein 14C (MW: 11641.7 Da, pI: 9.7), the Stefin 1 (MW: 11640.34 Da, pI: 5.86), the U6 snRNA-associated Sm-like protein LSm7 (MW: 11636.41 Da, pI: 5.1) and the FK506-binding protein 1B (MW: 11666.37 Da, pI: 8.64).

Using the same chip [WCX2 with 50 mM NaOAc (pH 4.5)] a protein of 14972.0 Da also exhibited an increase in expression due to aluminium on the 24 h profile (Fig. IV.18).

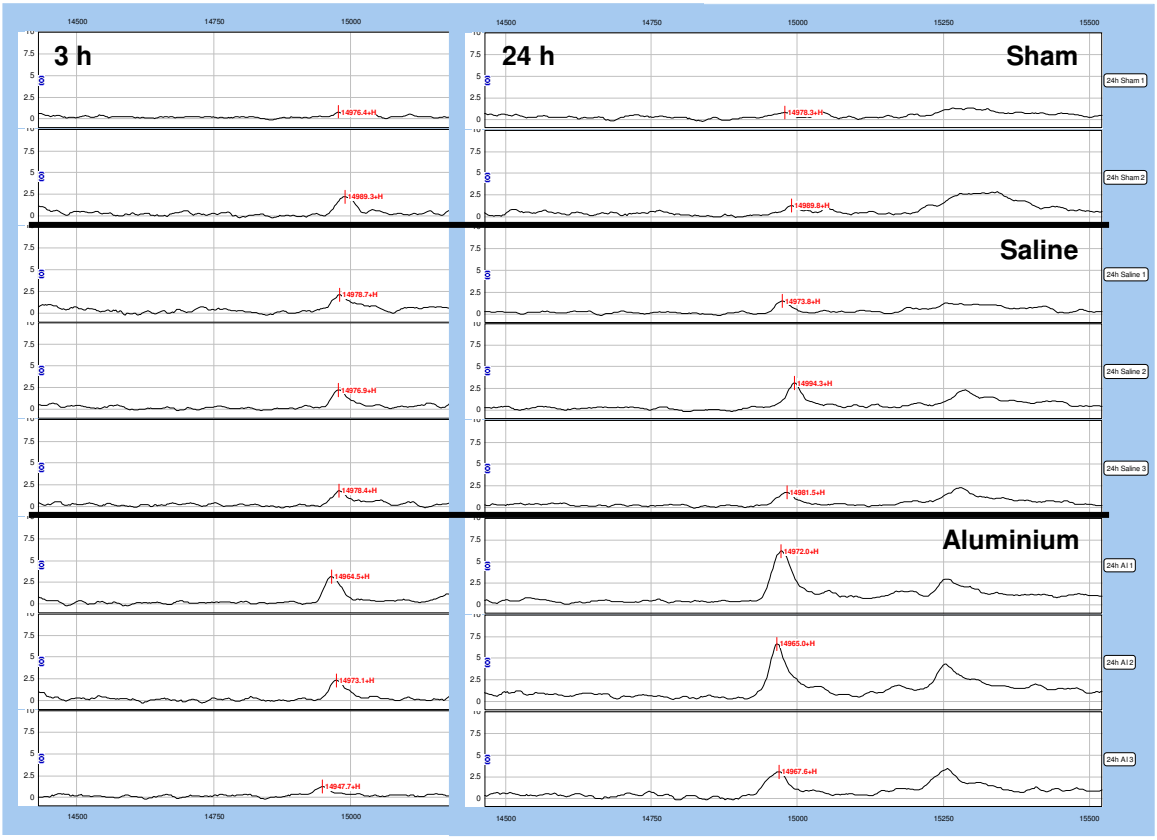

Fig. IV.18 – Detection of increased expression of a protein due to aluminium. The protein of 14972.0 Da was detected using the chip WCX2 with 50 mM NaOAc (pH 4.5).

Several putative proteins were identified in the database for this protein. The best candidate was the Glial cell line-derived neurotrophic factor precursor (MW: 14974.00 Da, pI: 9.44). Other possibilities were the Potassium voltage-gated channel subfamily E member 1-like protein (MW: 14968.21 Da, pI: 5.28), the Interleukin-17 precursor (MW: 14978.16 Da, pI: 8.84), the bone morphogenetic protein 5 precursor (MW: 14978.93 Da, pI: 7.77), the Interleukin-21 precursor (MW: 14964.48 Da, pI: 9.79) and the CD160 antigen precursor (MW: 14979.70 Da, pI: 6.39).

A protein of 15613.6 Da was also observed with increased aluminium-induced expression, but only at 24 h (Fig. IV.19).

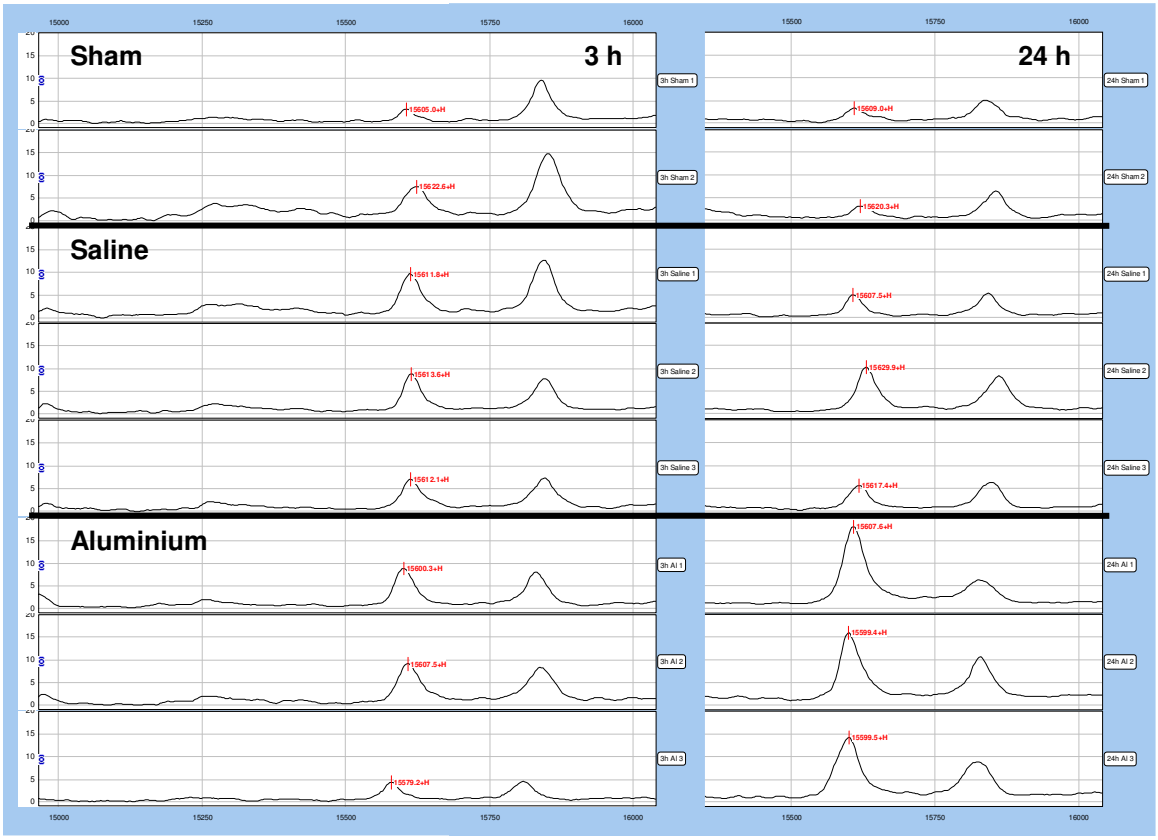

Fig. IV.19 - Aluminium-induced increased expression of a 15613.6 Da protein in mice. The protein was detected using the chip WCX2 with 50 mM NaOAc (pH 4.5).

Three putative proteins were identified in the data base for this protein: the SPUF protein precursor (secreted protein of unknown function) (MW: 15612.35 Da, pI: 4.79), the Microfibrillar-associated protein 5 precursor (MW: 15603.56 Da, pI: 5.61) and the 60S ribosomal protein L28 (MW: 15602.28 Da, pI: 12.02).

IV.4. SUMARY OF RESULTS

- *In vitro* studies:

- In PC12 cells, three peptides and one protein were detected with altered expression due to serum [one peptide (6710.6 Da, Fig. IV.3) showed increased levels of expression and two

peptides (3250.2 Da and 3780 Da, Fig. IV.4) and one protein (9940.4 Da, Fig. IV.5) revealed lower expression levels] and one protein (20245.5 Da, Fig. IV.6) exhibited decreased expression due to aluminium.

- In COS-1 cells, three proteins (7840.6 Da, 7967.1 Da and 8200.7 Da, Fig. IV.7) were discovered with decreased expression due to serum and two with altered expression due to aluminium [one with increased levels (10825.4 Da, Fig. IV.10) and the other with lower levels (9501.4 Da, Fig. IV.9)]. Two peptides (4377.8 Da and 4776.4 Da, Fig. IV.8A and 8B, respectively) were also identified with altered expression, apparently due to a double serum and aluminium decreasing effect.

- *In vivo* studies:

- In mice, five peptides were detected with decreased levels of expression due to aluminium (6160.8 Da, Fig. IV.12; 6556.9 Da and 6896.6 Da, Fig. IV.13; 4845.3 Da and 5232.4 Da, Fig. IV.15) and nine proteins with aluminium-induced altered expression [two with lower levels (7660.2 Da, Fig. IV.12; 13821.5 Da, Fig. IV.14) and seven with increased levels of expression (9805.9 Da, Fig. IV.11; 7103.1 Da and 7219.2 Da, Fig. IV.16; 11168.1 Da and 11644.5 Da, Fig. IV.17; 14972 Da, Fig. IV.18; 15613.6 Da, Fig. IV.19)].

- The putative proteins identified were very diverse with quite different functions.

- Aluminium may be implicated in a wide range of mechanisms.

IV.5. DISCUSSION

Surface-enhanced laser desorption/ionization (SELDI) time of flight (TOF) is a mass spectrometry technology for measuring the composition of a sampled protein mixture. Protein profiling has been applied to a variety of samples including plasma, urine, cerebrospinal fluid, saliva and solid tissue (Kasthuri *et al.*, 2006). This new proteomic technology has been useful providing information about the pathogenesis of many central and peripheral nervous system diseases (Johnson *et al.*, 2006), including AD by, for instance, monitoring Abeta peptides in cerebrospinal fluid of AD patients (Maddalena *et al.*, 2004) or finding biomarkers for schizophrenia (Lakhan *et al.*, 2006). Protein profile analysis is also increasingly used for identification of biomarkers of non-

neurological diseases as diverse as cancer (Ciordia *et al.*, 2006), diabetes (Puricelli *et al.*, 2006) or chronic lymphoid malignancies (Miguet *et al.*, 2006).

The form and speciation of aluminium is critical to its biological actions (Golub *et al.*, 2002). $AlCl_3$ is the most common inorganic aluminium salt used for *in vitro* studies. In solution, $AlCl_3$ dissociates and Al^{3+} is free to associate with a ligand from the culture medium, increasing its bioavailability. In the *in vivo* experiments a biological form of aluminium, aluminium lactate, was used for intraperitonial injections. Lactate, as well as citrate, maltolate or ascorbate, is one of the best aluminium ligands that allows aluminium to cross membranes *in vivo*. Indeed, it was reported that the uptake of aluminium was higher in mice injected intraperitonialy when it was administered in a complex form (aluminium-maltolate) than in an ionic form ($AlCl_3$) (Kaneko *et al.*, 2006).

One of the earliest reports of aluminium neurotoxicity was published by Scherp and Church (1937). These investigators described a characteristic neurological syndrome induced in experimental animals by a single injection of a minute amount of aluminium salt into the central nervous system. Within 20 days of injection, motor malfunction developed which was soon followed by severe convulsions and by subsequent death of the animal. In the terminal state, pathological examination revealed widespread cellular degeneration in various parts of the CNS accompanied by elevated levels of aluminium (Meiri *et al.*, 1993). Altered protein expression due to aluminium neurotoxicity has been reported. Strong and colleagues (1994) observed altered neurofilaments mRNA levels following acute aluminium neurotoxicity. Elevation of cerebral proteases after systemic administration of aluminium was also reported (Guo-Ross *et al.*, 1998). Infant rabbits exposed to aluminium maltolate in milk (lactating mother received aluminium injections) exhibited changes in brain protein synthesis which resemble those in infants injected directly with aluminium (Nicholls *et al.*, 1995).

In this study, various peptides and proteins were found to be up or down-regulated. However, due to unsolved technical problems related to the SELDI-TOF technology used it was only possible to detect peptides and low molecular weight proteins. In the *in vitro* studies some proteins were detected with altered expression due to serum withdrawal. Serum deprivation may induce changes conducting, for instance, to cell death. It was reported that serum deprivation induces cell death of PC12 cells (Kwon *et al.*, 2002) or of primary neuronal cultures (Hugon *et al.*, 2000). Thus, in this process, or others, some

proteins may be up or down-regulated. However, more interestingly, various proteins were discovered with altered expression due to aluminium. Several putative proteins for those discovered proteins have known functions in the most variable systems. Some of the putative proteins are related with mechanisms in which aluminium seems to induce toxicity. A putative protein for an aluminium up-regulated protein with 9.8 kDa (Fig. IV.11) is a cytokine B13 precursor. Putative proteins for a 14.9 kDa protein up-regulated by aluminium (Fig. IV.18) are the precursors of interkeukin-17 and interleukin-21. The aluminium-induced increase of other interleukin family members has been previously reported. The levels of interleukin-1alpha increased in the brains of animals following chronic aluminium exposure (Campbell *et al.*, 2004). Aluminium treatment of human glioblastoma cells caused an increase in the levels of interleukin-6 (Campbell *et al.*, 2002). The activity of apopain, an interleukin 1beta converting enzyme (ICE)-like cysteine protease was found increased following dosing with aluminium (Guo-Ross *et al.*, 1998). Elevation in the levels of pro-inflammatory cytokine interleukin-1beta induced by aluminium was also reported (Nedzvetsky *et al.*, 2006). Interleukins are a group of cytokines which are signalling compounds that, like hormones and neurotransmitters, are used extensively for inter-cell communication. Cytokines play a major role in a variety of immunological, infectious and inflammatory diseases. Chronic up-regulation of the innate immune responses in the central nervous system may jeopardize neuronal integrity through the prolonged production of toxic inflammatory mediators. Environmental exposures that further enhance the innate immune response may accelerate neurodegeneration. Environmental factors such as aluminium can trigger inflammatory events in the central nervous system (Campbel, 2004; Campbell *et al.*, 2002; Campbell *et al.*, 2004).

A putative protein for the 11.1 kDa protein which is up-regulated by aluminium (Fig. IV.17) is a death-associated protein 1 that may be related to apoptosis. This finding suggests that aluminium contributes to the augmenting of proteins leading to apoptosis. Indeed, apoptosis is known to be one of the consequences of aluminium toxicity (Savory *et al.*, 1999; Ghribi *et al.*, 2001b; Yang *et al.*, 2004; Johnson *et al.*, 2005). Interestingly, a putative protein for the 4.8 kDa peptide found down-regulated by aluminium (Fig. IV.15) is the cytochrome *c* oxidase polypeptide VIII-liver, mitochondrial precursor. This suggests a possible involvement of aluminium in mitochondria impairment. In fact, it was reported that following chronic aluminium exposure the enzymatic activity of the terminal enzyme

of the electron transport chain cytochrome oxidase was found significantly decreased (Kaur and Gill, 2006). Moreover, mitochondrial cytochrome *c* oxidase subunit III was detected selectively down-regulated by aluminium exposure (Bosetti *et al.*, 2001). Mitochondrial dysfunction has been implicated in neuronal cell death, namely the opening of the mitochondrial permeability transition pore and the cytochrome *c* release which are associated with apoptosis. Aluminium has been related with this mitochondrial impairment leading to apoptosis (Ghribi *et al.*, 2001a; Savory *et al.*, 2003; Griffioen *et al.*, 2004; Johnson *et al.*, 2005). APP gamma-secretase C-terminal fragment 59 is a putative protein for the 6.89 kDa protein down-regulated by aluminium. Gamma-secretase cleaves APP leading to the formation of the Abeta peptide. It seems that aluminium down-regulation of this enzyme might inhibit APP processing. In fact, APP accumulation in damaged neuronal processes and microglia was observed following intracerebral administration of aluminium salts in rat brain (Shigematsu and McGeer, 1992). All these aluminium-induced altered proteins are associated with mechanisms through which aluminium exerts toxicity leading to neurodegeneration.

CHAPTER V

CONCLUDING REMARKS

V. CONCLUDING REMARKS

Excessive aluminium exposure impairs neurocognitive function in humans and animals (Gotow *et al.*, 1995; Savory *et al.*, 1999; Miu *et al.*, 2003; Roig *et al.*, 2006). Epidemiologic studies have shown a potential link between chronic aluminium exposure and neurodegenerative diseases, such as dialysis encephalopathy (Alfrey *et al.*, 1976; Savory and Wills, 1984; Kerr and Ward, 1988), ALS/PD complex of Guam (Perl *et al.*, 1982; Kurland, 1988) or AD (Perl and Brody, 1980; Perl, 1988; Xu *et al.*, 1992a). Indeed, aluminium exposure and apoptotic cell death have been implicated in neurodegeneration. The mechanisms by which this metal affects the nervous system are poorly understood, but it is known to influence gene expression, alter protein phosphorylation and inhibit some cellular enzymes (Li *et al.*, 1998). There is also evidence for aluminium-induced chromosomal aberrations, micronuclei in human lymphocytes (Banasik *et al.*, 2005), DNA damage and inhibition of DNA repair (Lankoff *et al.*, 2006). Aluminium was shown not only to induce apoptosis via generation of DNA damage, but also through activation of several apoptotic signalling mechanisms including nuclear translocation of *gadd*153, triggering NF-kB signalling, up-regulation of pro-apoptotic factors such as Bax, down-regulation of the anti-apoptotic factor Bcl-2 and activation of caspase-3 and caspase-12 (Ghribi *et al.*, 2001b, Savory *et al.*, 2003; Yang *et al.*, 2004; Johnson *et al.*, 2005). Thus, apoptosis seems to be the final consequence of aluminium toxicity.

It is well known that aluminium, a trivalent cation unable to undergo redox reactions, has the ability to promote the pro-oxidant properties of transition metals such as iron (Gutteridge *et al.*, 1985; Oteiza, 1994; Xie *et al.*, 1996). In parallel, the decrease of MTT reduction following aluminium exposure suggests modification of mitochondrial function. Alterations in mitochondrial function may lead to excess ROS production, which in turn can lead to oxidative injury. Since mitochondria are rich in iron one may speculate that the iron-dependent lipid peroxidation potentiated by aluminium-induced ROS production is one of the mechanisms at the onset of aluminium toxicity. These observations are supported by several reports. Indeed, subcellular analysis revealed that aluminium injections into rat brain resulted in its significant enrichment in the

mitochondrial fraction (Ogasawara *et al.*, 2003). Additionally, following neuronal aluminium exposure, mitochondrial ultrastructure analysis showed that aluminium appears to impair the mitochondrial membrane and cristae, as increased cell death rate, enhanced ROS, decreased mitochondrial membrane potential, and decreased enzyme activity in mitochondria (MTT reduction) were observed (Niu *et al.*, 2005). Furthermore, following chronic aluminium exposure the enzymatic activity of cytochrome oxidase (the terminal enzyme of the electron transport chain) was significantly decreased (Kaur and Gill, 2006). Thus, a potential mechanism through which aluminium exerts neurotoxicity involves mitochondria impairment and consequently oxidative damage and cell death. Interestingly, cell death due to oxidative injury is strongly suspected to be a contributory factor in many neurological diseases.

The most significant effect of aluminium on the central nervous system of experimental animals is neurodegeneration, related to neurofibrillary degeneration. The latter is characterized by the accumulation of neuronal perikarial aggregates of hyperphosphorylated neurofilaments. Erasmus and co-workers (1993) suggested that aluminium binds to soluble monomeric or oligomeric neurofilament proteins via a phosphate group, resulting in abnormal neurofilament assembly, impaired axonal transport, and subsequent aggregation. Reduction of neurofilament axonal transport during aluminium neurotoxicity (Shea *et al.*, 2003) may have serious implications for memory and learning. In fact, aluminium-induced impairment of hippocampal long-term potentiation, a model for synaptic plasticity underlying some forms of learning and memory, has been reported in rats both *in vivo* and *in vitro* (Platt *et al.*, 1995), suggesting that aluminium can lead to memory impairment.

PP1, the "forgetfulness molecule", is necessary for maintaining long-term depression (Morishita *et al.*, 2001) and has been associated with age-related memory and learning deficits (Genoux *et al.*, 2002). It is possible that some of the toxic effect of aluminium might also be mediated via its effect on PP1 and/or neurofilaments. In this work, we observed that aluminium induced down-regulation of the expression and activity of both PP1 isoforms studied. As protein phosphatases are key players in the intracellular signal transduction pathways, this alteration may lead to an imbalance in cellular protein phosphorylation systems and subsequently to neurodegeneration. Up to now, aluminium has been shown to impair intracellular signal transduction involving G-protein-mediated

pathways and phosphoinositide-producing systems (Shafer *et al.*, 1994; Golub *et al.*, 2002), as well as to interfere with intracellular calcium homeostasis (Shi and Haug, 1992; Haug *et al.*, 1994; Kaur and Gill, 2005). Furthermore, as this cation induces conformational changes in proteins and strongly binds to the phosphate groups of proteins, phosphorylated proteins, such as protein phosphatases and specially their regulatory subunits, constitute excellent aluminium targets. Using different approaches, we have also demonstrated that aluminium also induced alterations in the expression of other proteins. However, those aluminium-induced changes probably occurred through different mechanisms of action.

In this work we demonstrated aluminium-induced toxicity in two cell lines of different lineage and in primary neuronal cultures, with concomitant down-regulation of protein phosphatase expression and activity. The specificity of these effects was suggested by their reversion following aluminium withdrawal. Moreover, in primary neurons aluminium exposure resulted in reduced expression of synaptophysin and neurofilaments, although it did not alter protein phosphatase levels. Furthermore, our *in vivo* studies involving injection of aluminium directly into mouse brains led to alterations in the expression levels of several peptides. Interestingly, bioinformatics analysis indicates that some of these putative proteins are associated with apoptosis, mitochondria impairment and inflammatory mechanisms.

Various interconnected hypotheses have been advanced that underscore different events as a rationale for the initiation of the aluminium toxic insult to the brain. Nonetheless, no single unifying mechanism of aluminium neurotoxicity has been identified. Although the relation between aluminium and the pathogenesis of neurodegenerative diseases remains controversial, new lines of evidence make it difficult to contradict and numerous studies support an "aluminium hypothesis" (Flaten, 2001; Zatta *et al.*, 2003; Gupta *et al.*, 2005; Kawahara, 2005). Furthermore, it is widely accepted that aluminium is a powerful neurotoxicant, and can cause cognitive deficiency and dementia when it enters the brain. The results presented here may predict that this metal induces cellular toxicity through mechanisms that lead to apoptosis, most probably via mitochondrial impairment. In addition, the hypothesis that aluminium may be a cause of neuritic degeneration and neuronal loss was supported by the observed alterations in neuronal morphology and simultaneous down-regulation of neurofilament proteins and

synaptophysin. Concomitantly, aluminium impaired PP1 activity and expression, an enzyme that is a central player in many neuronal signal transduction pathways, contributing therefore to abnormal neuronal protein phosphorylation. To date more than twenty primary neuronal signaling cascades have been shown to be under the regulation of the PP1/DARPP-32 system in the striatum or the PP1/I1 system in other brain regions. Interestingly, PP1 has also been suggested to play a key central role in the molecular mechanisms underlying the actions of several drugs of abuse. Finally, by altering the expression of proteins associated with mechanisms as diverse as apoptosis and inflammation, aluminium may also induce substantial cellular toxicity via different pathways, further underscoring the difficulty in defining a single mechanism to explain aluminium neurotoxicity.

The major contribution of this work was to provide evidence that aluminium neurotoxicity is linked to the down-regulation of PP1 expression and activity, an effect that may ultimately lead to alterations in neuronal phosphorylation systems and thus to the abnormal hyperphosphorylation of key proteins. From the data presented in this dissertation it may be concluded that the study of aluminium neurotoxicity remains current and of major importance concerning its implications for human exposure.

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APPENDIX

APPENDIX

APPENDIX I - Solutions

CELL LINES AND RAT CORTICAL PRIMARY CULTURES

Cells seeding, maintenance and experiments:

Complete RPMI 1640 medium (PC12 cells)

For a final volume of 1 L, dissolve one pack of RPMI 1640 powder (Gibco, Invitrogen) in deionised H_2O and add:

- $NaHCO_3$ (Sigma-Aldrich) 0.85 g
- Horse Serum (HS) 100 ml (10% v/v)
- Foetal Bovine Serum (FBS) 50 ml (5% v/v)
- Antibiotic/antimycotic solution 10 ml (1% v/v)

Adjust to pH 7.4, sterilize by filtering through a 0.2 μm filter and store at 4 ºC.

Notes:

- Antibiotic/antimycotic solution (100x) (Gibco, Invitrogen) contains 10,000 U/ml Penicillin, 10,000 μg/ml Streptomycin, 25 μg/ml Amphotericin B in 0.85% saline.
- HS and FBS (Gibco, Invitrogen) are heat inactivated for 30 min at 56 ºC.

RPMI 1640 without phosphates medium (PC12 cells)

Using one bottle of 500 ml RPMI 1640 without phosphates (liquid) (Biosource) add:

- Antibiotic/antimycotic solution 10 ml

Sterilize by filtering through a 0.2 μm filter and store at 4 ºC.

Complete DMEM medium (COS-1 cells)

For a final volume of 1 L, dissolve one pack of DMEM powder (with L-glutamine and 4500 mg glucose/L, Sigma-Aldrich) in deionised H_2O and add:

- $NaHCO_3$ 3.7 g
- Foetal Bovine Serum (FBS) 100 ml (10% v/v)
- Antibiotic/antimycotic solution 10 ml

Adjust to pH 7.4, sterilize by filtering through a 0.2 μm filter and store at 4 ºC.

DMEM without phosphates medium (COS-1 cells)

For a final volume of 1 L, dissolve one pack of DMEM powder (Sigma-Aldrich) in deionised H_2O and add:

- $NaHCO_3$ 3.7 g
- 20 mM HEPES (Sigma-Aldrich) 5.2 g
- Antibiotic/antimycotic solution 10 ml

Adjust to pH 7.4, sterilize by filtering through a 0.2 μm filter and store at 4 ºC.

PBS (1x)

For a final volume of 500 ml, dissolve one pack of BupH Modified Dulbecco's Phosphate Buffered Saline Pack (Pierce) in deionised H_2O. Final composition:

- Sodium Phosphate 8 mM
- Potassium Phosphate 2 mM
- NaCl 140 mM
- KCl 10 mM

Sterilize by filtering through a 0.2 μm filter and store at 4 ºC.

Complete Neurobasal medium (primary neuronal cultures)

This serum-free medium (Neurobasal, Gibco, Invitrogen) is supplemented with:

- B27 supplement (Gibco, Invitrogen) 2%
- L-glutamine (Gibco, Invitrogen) 0.5 mM
- Glutamate (Gibco, Invitrogen) 25 μM
- Gentamicine (Gibco, Invitrogen) 50 μg/ml
- Phenol Red (Sigma-Aldrich) 0.001%

Adjust to pH 7.4, sterilize by filtering through a 0.2 μm filter and store at 4 ºC.

Hank's Balanced Salt Solution (HBSS) (primary neuronal cultures)

This salt solution is prepared with deionised H_2O. Final composition:

- NaCl	137 mM
- KCl	5.36 mM
- KH_2PO_4	0.44 mM
- $Na_2HPO_42H_2O$	0.34 mM
- $NaHCO_3$	4.16 mM
- Glucose	5 mM
- Sodium Pyruvate	1 mM
- HEPES	10 mM

Adjust to pH 7.4, sterilize by filtering through a 0.2 μm filter and store at 4 ºC.

Aluminium solutions:

Aluminium solution (for cells exposure)

Aluminium solution is prepared fresh for each experiment.

Prepare 10 mM stock solution by dissolving 0.12 mg $AlCl_3.6H_2O$ (Sigma-Aldrich) in 5 ml 50 mM HEPES, pH 7.4.

Dilute 10x with the same buffer and filter (0.2 μm) to sterilize.

Dilute 10 mM aluminium stock solution in RPMI or DMEM without phosphates to the experimental concentrations.

Aluminium solution (for recombinant PP activity assays)

Prepare 1 mM $AlCl_3.6H_2O$ in Tris, pH 9.

Dilute in 50 mM Tris-HCl, pH 7.5 containing 0.1 mM EGTA, 0.03% (v/v) Brij-35 and 0.1% (v/v) 2-mercaptoethanol to the working concentrations.

Cells Fixation:

1 mg/ml Poly-L-ornithine stock solution (10x) (100 ml)

To a final volume of 100 ml, dissolve in deionised H_2O 100 mg of poly-L-ornithine (Sigma-Aldrich). Sterilize by filtering through a 0.2 µm filter and store at -20 ºC.

Prior to use, dilute the stock solution 10x with deionised H_2O.

10 mg/ml Poly-D-lysine stock solution (100x) (10 ml)

To a final volume of 10 ml, dissolve in borate buffer 100 mg of poly-D-lysine (Sigma-Aldrich). Sterilize by filtering through a 0.2 µm filter.

Poly-D-lysine solution (100 ml)

To a final volume of 100 ml, dilute 1 ml of the 10 mg/ml poly-D-lysine stock solution in borate buffer.

Borate buffer (1 L)

To a final volume of 1 L, dissolve in deionised H_2O 9.28 g of boric acid (Sigma-Aldrich). Adjust to pH 8.2, sterilize by filtering through a 0.2 µm filter and store at 4 ºC.

PROTEINS MANIPULATION

SDS-PAGE:

LGB (Lower gel buffer) (4x) (1 L)

- Tris	181.65 g
- SDS	4 g

Dissolve in deionised H_2O, adjust the pH to 8.9 with HCl and adjust the volume to 1 litre.

UGB (Upper gel buffer) (5x) (1 L)

Dissolve 75.7 g of Tris base in deionised H_2O, adjust the pH to 6.8 with HCl and adjust the volume to 1 litre.

30% Acrylamide/0.8% Bisacrylamide solution (100 ml)

- Acrylamide 29.2 g
- Bisacrylamide 0.8 g

Dissolve in deionised H_2O and adjust the volume to 100 ml. Filter through a 0.2 μm filter and store at 4ºC.

10% APS (ammonium persulfate) (10 ml)

In 10 ml of deionised H_2O dissolve 1g of APS. Note: prepare fresh before use.

10% SDS (sodium dodecilsulfate) (10 ml)

In 10 ml of deionised H_2O dissolve 1g of SDS.

Loading (sample) gel buffer (4x) (10 ml)

- 1M Tris solution (pH 6.8) 2.5 ml (250mM)
- SDS 0.8 g (8%)
- Glicerol 4 ml (40%)
- β-Mercaptoetanol 2 ml (20%)
- Bromofenol blue 1 mg (0.01%)

Adjust the volume to 10 ml with deionised H_2O. Store in darkness and at RT.

1M Tris (pH 6.8) solution (250 ml)

Dissolve 30.3 g of Tris base in deionised H_2O, adjust pH to 6.8 and adjust final volume to 250 ml.

Running buffer (10x) (1 L)

- Tris 30.3 g (250 mM)
- Glycine 144.2 g (2.5 M)
- SDS 10 g (1%)

Dissolve in deionised H_2O, adjust the pH to 8.3, and adjust the volume to 1 litre.

15 cm gels:

Resolving (lower) gel solution (60 ml)	**7.5%**	**12%**
- H_2O	29.25 ml	20.7 ml
- 30% Acryl/0.8% Bisacryl solution	15.0 ml	24.0 ml
- LGB (4x)	15.0 ml	15.0 ml
- 10% APS	300 µl	300 µl
- TEMED	30 µl	30 µl

Stacking (upper) gel solution (20 ml)	**3.5%**
- H_2O	13.2 ml
- 30% Acryl/0.8% Bisacryl solution	2.4 ml
- UGB (5x)	4.0 ml
- 10% SDS	200 µl
- 10% APS	200 µl
- TEMED	20 µl

Immunoblotting solutions:

Electrotransfer buffer (1x) (1 L)

- Tris	3.03 g (25 mM)
- Glycine	14.41 g (192 mM)

Dissolve in deionised H_2O, adjust the pH to 8.3 with HCl and adjust the volume to 800 ml with deionised H_2O. Just prior to use add 200 ml of methanol (20%).

TBS (Tris Buffered Saline) (10x) (1 L)

- Tris	12.11 g (10 mM)
- NaCl	87.66 g (150 mM)

Dissolve in deionised H_2O, adjust the pH to 8.0 with HCl and adjust the volume to 1 litre.

TBS-T (Tris Buffered Saline + Tween) (10x) (1 L)

- Tris 12.11 g (10 mM)
- NaCl 87.66 g (150 mM)
- Tween 20 5 ml (0.05%)

Dissolve in deionised H_2O, adjust the pH to 8.0 with HCl and adjust the volume to 1 litre.

Blocking solution (100 ml)

- TBS-T stock solution (10x) 10 ml
- non-fat milk (dry powder) 5 g

Dissolve in deionised H_2O and adjust volume to 100 ml.

Antibody solution (25 ml)

- TBS-T stock solution (10x) 2.5 ml
- non-fat milk (dry powder) 0.75 g

Dissolve in deionised H_2O and adjust volume to 25 ml. Add antibody, mix gently without vortex, and store at -20 ºC.

Alkaline Phosphatase (AP) reaction solution (1 L)

- Tris-HCl (pH 9.5) 12.11 g (100 mM)
- NaCl 5.85 g (100 mM)
- $MgCl_2$ 1.02 g (5 mM)

Dissolve Tris base in deionised H_2O and adjust solution to pH 9.5 with HCl. Dissolve the other solutes and adjust volume to 1 litre.

AP stop solution (1 L)

- Tris-HCl (pH 9.5) 2.42 g (20 mM)
- EDTA 1.86 g (5 mM)

Dissolve Tris base in deionised H_2O and adjust with HCl to pH 9.5. Add EDTA after and adjust volume to 1 litre.

ASSAY OF PROTEIN PHOSPHATASES

50 mM Tris / 0.1 mM EDTA solution

For a final volume of 15 ml add to deionised H_2O from respective stocks:

	Final concentration	Stock	Volume (15 ml)
- Tris-HCl pH 7.5	50 mM	250 mM	3 ml
- EDTA	0.1 mM	1 mM	1.5 ml
- H_2O			10.5 ml

Homogenization buffer

For a final volume of 4 ml add to deionised H_2O from respective stocks:

	Final concentration	Stock	Volume (4 ml)
- Tris-HCl pH 7.5	50 mM	250 mM	800 µl
- EDTA	0.1 mM	1 mM	400 µl
- EGTA	0.1 mM	1 mM	400 µl
- NaCl	150 mM	1.5 M	400 µl
- DTT	0.1 mM	2 mM	200 µl
- Benzamidine	1 mM	200 mM	20 µl
- PMSF	0.1 mM	100 mM	4µl
- Leupeptin	5 µg/ml	0.5 mg/ml	4µl
- dH_2O			1772 µl

Preparation of ^{32}P-labelled phosphorylase *a*

Buffer A

- Na-2-glycerophosphate, pH 7.5	50 mM
- Glycerol	10% (v/v)
- EGTA	0.1 mM
- 2-mercaptoethanol	0.1% (v/v)
- Microcystin	0.1 µM

Add 2-mercaptoethanol to buffers just before use. For 1 ml of buffer A, add 2 µl of 50 µM microcystin and 1 µl of 2-mercaptoethanol to 997 µl of pre-prepared buffer containing the remaining components.

Buffer B

- 125 mM Na-2-glycerophosphate, pH 8.6

Buffer C

- 50 mM Tris-HCl, pH 7.0
- 0.1% (v/v) 2-mercaptoethanol

Buffer D

- 10 mM Tris-HCl, pH 7.0
- 0.1% (v/v) 2-mercaptoethanol

Buffer E

- 50 mM Tris-HCl, pH 7.0

Phosphorylase kinase (20 mg/ml in buffer A)

Phosphorylase *b* (100 mg/ml in buffer A)

90% saturated ammonium sulphate

Add 475 g per litre, adjust pH to 7.0 with NH_4OH. The ammonium sulphate is added to the water slowly in order to dissolve till saturation.

50 µM Microcystin

Prepare in DMSO and keep aliquots in -20ºC.

Assay of phosphorylase phosphatase (PP1 and/or PP2A) activity

Buffer A

- Tris-HCl, pH 7.5	50 mM
- EGTA	0.1 mM
- 2-mercaptoethanol	0.1%
- BSA	1 mg/ml
- $MnCl_2$	1mM

Buffer B

- Tris-HCl, pH 7.5	50 mM
- EGTA	0.1 mM
- 2-mercaptoethanol	0.1%
- Brij-35	0.03%

Buffer C

As buffer A, but without BSA.

75 mM Caffeine

Adjust pH to 7.0 and store in the dark at RT (caffeine is degraded by light and will crystallize if stored at 4 ºC).

CIPHERGEN PROTEINCHIP SOLUTIONS

All solutions are prepared with HPLC water.

Aluminium lactate solution (mice injections)

Dissolve in 1 ml saline solution 200 mg of Aluminium lactate (Sigma-Aldrich).

0.5% OGP/PBS solution

Prepare 10% OGP (n-octyl glucopyranoside, Sigma-Aldrich) in deionised H_2O by dissolving 1 g OGP in 10 ml H_2O. Dilute 20x by adding 500 µl 10% OGP to 9.5 ml PBS.

100 mM Cupric sulfate ($CuSO_4$) solution

To a final volume of 25 ml, add 0.399 g to 25 ml HPLC H_2O.

50 mM HEPES pH 7.4

To a final volume of 100 ml, add 1.19 g HEPES to 80 ml HPLC H_2O, adjust to pH 7.4 and adjust the volume to 100 ml.

50% Acetonitrile (ACN)

To a final volume of 50 ml, add 25 ml 100% ACN to 25 ml HPLC H_2O.

PBS

Dissolve 1 package (PBS, Pierce) in 1000 ml HPLC H_2O.

PBS/0.5 M NaCl

To a final volume of 500 ml, add 14.61 g NaCl to 400 ml PBS. Mix and adjust volume to 500 ml.

PBS/25% ACN

To a final volume of 500 ml, add 125 ml ACN (100%) to 375 ml PBS.

50 mM Tris pH 9.0

To a final volume of 500 ml, add 25 ml from stock 1 M pH 8.0 to 475 ml HPLC H_2O, adjust to pH 9.0.

50 mM Sodium acetate solution pH 4.5

To a final volume of 500 ml, add 2.05 g sodium acetate to 500 ml HPLC H_2O, adjust to pH 4.5.

EAM molecule solution

The solvent system for EAM (Energy Absorbing Molecule) and the EAM solution itself must be prepared fresh every day. Acetonitrile (ACN) volatilizes rapidly and EAMs break down significantly within 24 hours in solution at RT.

A saturated solution is prepared so some pellet can be visualized, use only the supernatant.

- Weight 5 mg Sinapinic acid (SPA, Sigma) in a microfuge tube (preferably brown or covered with foil, to protect from light).
- Add 100 µl of a freshly prepared 1:1 solution of 100% ACN and 1% trifluoroacetic acid (TFA) (final concentration: 50% ACN and 0.5% TFA) and strongly vortex.

APPENDIX II – Kits and methods

CELL CULTURE

PC12 cell culture maintenance

PC12 cells were cultured in RPMI 1640 medium (Gibco, Invitrogen) supplemented with 10% (v/v) heat-inactivated horse serum (Gibco, Invitrogen), 5% (v/v) heat-inactivated fetal bovine serum (Gibco, Invitrogen), 1% (v/v) antibiotic-antimycotic solution (100 U/ml Penicillin, 100 µg/ml Streptomycin, 0.25 µg/ml Amphotericin B) (Gibco, Invitrogen) and 0.85 g/L $NaHCO_3$. Routinely, cells were plated in 100 mm plates and maintained at 37 ºC in an atmosphere of 5% CO_2. Cells were subcultured whenever ~95% confluency was reached (every 3-5 days). For experimental procedures, cells were collected, counted and plated onto 6-well plates that were previously treated with 100 µg/ml poly-L-ornithine (Sigma-Aldrich) for 10 minutes and washed 3 times with sterile deionised water.

COS-1 cell culture maintenance

COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Invitrogen), 1% (v/v) antibiotic-antimycotic solution and 3.7 g/L $NaHCO_3$. Cells were routinely plated in 100 mm cell culture dishes and grown in a humidified incubator at 37 ºC and 5% CO_2. Whenever ~95% confluency was reached (every 3-5 days) cells were subcultured and for successful dissociation trypsin-EDTA (Gibco, Invitrogen) was used. For experimental procedures, cells were collected, counted and seeded onto 6-well plates.

ANIMAL PROCEDURES

Rat cortical primary cultures

Rat cortical neurons were dissociated from cortex of Wistar Hannover 18 days rat embryos whose mother was killed by rapid cervical dislocation. After cortex dissection, tissues were treated for 10 min at 37 ºC with a 0.45 mg/ml trypsin/0.18 mg/ml deoxyribonuclease solution in Ca^{2+}- and Mg^{2+}-free HBSS, supplemented with BSA (Merck, VWR International). Cells were washed with HBSS supplemented with 10% FBS to stop trypsinization, centrifuged at 200 g for 3 min, and further washed and centrifuged with HBSS for serum withdraw. Cells pellet was ressuspended in complete Neurobasal medium, which is supplemented with 2% B27.. Viability and cellular concentration were assessed by using the Trypan Blue excluding dye (0.4% Trypan Blue solution, Sigma-Aldrich), and cells with (dead) or without (living) intracellular blue staining were counted in a hemocytometer chamber. Cellular viability was calculated and normally higher than 95%. These neuronal cells were finally plated at 0.9 x 10^6 cells/well in 100 µg/ml poly-D-lysine pre-coated six-well plates. Cells were maintained in 2 ml of complete Neurobasal medium in a humidified incubator at 37 ºC and 5% CO_2. Three and seven days after plating, 500 µl of cultured medium was replaced with 500 µl of glutamate-free complete Neurobasal medium. For epifluorescence analyzis, cells were plated on 100 µg/ml poly-D-lysine pre-coated glass coverslips in six-well plates.

Mice aluminium injected samples

C57BL/6J mice, females with 9 months, were weighted and anaesthetized with Ketamine 150 mg/Kg (injected intramuscularly) and Xylazine 10 mg/Kg (injected intraperitoneally). A few minutes after, the skin from their head was cut and they were injected with one dose of aluminium lactate solution (1 µl containing 200 µg aluminium or 680 pmoles, 0.68 mM) in the left frontoparietal cortex area (1.5 mm deep). Control groups of mice injected with saline solution (Saline) or just touched with a needle (Sham) in the same cortex area were also preformed. Each group had at least four animals. After injection, the skin was stapled and the animals were allowed to recover for 3 or 24 hours before being sacrificed by decapitation. The cortex was removed and the puncted surrounding area (4-5 mm^3) was isolated and frozen in dry ice and stored at -80 ºC. All

samples were homogenized in 50 µl PBS/0.5% OGP and sonicated for 20 seconds. Protein concentration was estimated with BCA protein assay and adjusted to 3 µg/µl in PBS/0.5% OGP solution in a final volume of 40 µl (120 µg of total protein). Samples were diluted 1:3 in respective binding buffers.

VIABILITY ASSAYS

Trypan blue assay

This method is based on the exclusion dye Trypan blue (Sigma-Aldrich). Cells that became blue after incubated with the dye are dead cells (the cellular membrane is affected and allows the dye to go inside) on the other hand, cells that remains uncoloured are living cells. From the cellular suspension 90 µl was removed, added to 10 µl of 0.4% Trypan blue solution and mixed for 1 minute. Ten µl of that mixture was loaded onto a hemocytometer chamber and cells were counted using an inverted optical microscope.

MTT assay

This method is based on the reduction of MTT, a water soluble tetrazolium salt, by mitochondrial dehydrogenase, to an insoluble intracellular purple formazan. The extent of reduction of MTT was measured spectrophotometrically at 570 nm, according to Mossman (1983). After cells treatment, the conditioned medium was removed and 0.5 mg/ml MTT (Sigma-Aldrich) solution (in serum-free RPMI or serum-free DMEM) was added and incubated for 3 hours at 37 ºC. The resulting insoluble formazan precipitates were solubilized with 0.04 M HCl/Isopropanol. The absorbance of the converted dye was measured at a wavelength of 570 nm in a Cary 50 spectrophotometer. The cellular viability was expressed as a percentage of O.D. values of control cells, meaning that 90% of viability denotes a decrease of 10% on viability.

PROTEINS MANIPULATION

Protein Assay kit (BCA)

Samples total protein measurements were performed with BCA protein assay kit (Pierce), following the manufacturer's instructions. The method combines the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (the biuret reaction) with a sensitive colorimetric detection of the Cu^{+} cation using a reagent containing bicinchoninic acid (BCA). BCA complexes with Cu^{+} and produces a purple colour, which exhibit a strong absorbance at 562 nm.

The total protein from each sample was measured using 50 µl of cell lysate in 1% SDS (or 25 µl of cell lysate plus 25 µl of 1% SDS). Standards were prepared with different volumes of 2 mg/ml bovine serum albumin (BSA) solution (Pierce) (with known final concentrations of 2-80 µg) and final volumes adjusted to 50 µl with 1% SDS. One ml of working reagent (WR) was added to each sample or standard, and mixed well. WR was previously prepared with 50:1 of reagent A (sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.2 N sodium hydroxide) and reagent B (4% cupric sulfate). Samples and standards were incubated at 37 ºC for 30 minutes. Temperature from samples and standards was let to cool to RT and absorbance was read at 562 nm in a spectrophotometer Cary 50 (Varian). A standard curve was prepared by plotting BSA standard absorbance vs. BSA concentration and used to determine the total protein concentration of each sample.

SDS-PAGE (for Western blotting)

SDS polyacrylamide gel electrophoresis (SDS-PAGE) separations were carried out using well established methods (Laemmli, 1970), where proteins are separated by their molecular weight and negative charge due to SDS-amino acid binding. The gels percentage and size chosen depend on the molecular weight of the proteins to be separated in the gel. Gels were prepared by mixing several components (Appendix I). The resolving gel solution was immediately and carefully pipetted down the spacer into the gel sandwich, leaving free space for the stacking gel. Water was carefully added to cover the top of the gel and the gel was allowed to polymerize for 1 h. Stacking gel solution was prepared according to Appendix I. The water was poured out and the stacking gel was added to the

gel sandwich; a comb was inserted and the gel allowed to polymerize for 1 h. In parallel, samples were prepared by adding to the protein sample solution ¼ volume of 4x LB (Loading Buffer). Samples microtubes were boiled and spinned down, the combs removed and the gels wells filled with Tris-Glycine running buffer. The samples were carefully loaded into the wells, and electrophoretically separated using a 90 mA electric current. Molecular weight markers (Kaleidoscope Prestained Standards or Prestained SDS-PAGE Standards – Broad Range, BioRad) were also loaded and resolved side-by-side with the samples. In some experiments, as the number of samples was more than the number of wells, samples were loaded into two similar gels and a control sample was loaded in both gels.

Proteins electrotransfer

Western blotting was carried out by direct electrotransfer the proteins, that were electrophoretically separated by SDS-PAGE, from the gel to a nitrocellulose membrane, while keeping their position (Burnette, 1981). 3MM blotter papers and a nitrocellulose membrane were used to build up the transfer sandwich. The gel was removed from the electrophoresis device and the stacking gel discarded. A transfer sandwich was assembled under transfer buffer, in the following order: sponge, 3MM blotter paper, gel, nitrocellulose membrane, 3MM paper, sponge. The cassette was placed in the transfer device, previously filled with transfer buffer, oriented so that the negatively charged proteins migrate towards the anode. Electrotransfer was let to proceed for 18 h at 200 mA, after what the membrane was allowed to dry on a clean paper.

Immunological detection

Proteins electrotransfered to nitrocellulose membrane can be immunologically detected. Initially, membranes were soaped in 1x TBS for 5 min. Blocking of possible non-specific binding sites of the primary antibody was performed by immersing the membrane in 5% (w/v) non-fat dry milk (Johnson *et al*., 1984) in 1x TBS-T solution for 1 h. Further incubation with primary antibody was carried out for the specified times, ranging from 2 h to overnight incubation at 4 ºC with agitation. After three washes with 1x TBS-T, of 10 min each, the membrane was further incubated with the appropriate secondary antibody for 2 h with agitation. All primary and secondary antibodies used were

diluted in 1x TBS-T/non-fat dry milk (3% w/v) at the dilutions specified in Chapter II and III (Table II.1 and III.1). Membranes were additional washed three times with 1x TBS-T, before being submitted to one of the following detection methods.

Enhanced chemiluminescence detection (ECL and ECL+ kits)

Enhanced chemiluminescence (ECL) (Amersham Pharmacia) is a light emitting non-radioactive method for detection of immobilised specific antigens, conjugated directly or indirectly with horseradish peroxidase-labelled antibodies. The ECL reaction is based on the oxidation of the cyclic diacylhydrazide luminal (Whitehead *et al*., 1979). In a dark room, the membrane was incubated for 1 min at RT with the ECL detection solution or for 5 min with the ECL+ detection solution. These solutions were prepared fresh following the manufacturer's instructions. The membrane was wrapped in cling-film and exposed to an autoradiography film (X-Omat, Kodak, Sigma-Aldrich) inside a film cassette (Kodak, Sigma-Aldrich). The film was developed in a developing solution (Kodak, Sigma-Aldrich), washed in water and fixed in a fixing solution (Kodak, Sigma-Aldrich).

Colorimetric detection (NBT/BCIP)

The substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (NBT/BCIP) (Promega) is converted in situ into a dense purple compound by immuno-localized alkaline phosphatase. Each 15 cm membrane was developed by adding to it a solution of 66 µl of NBT and 33 µl of BCIP in 10 ml of Alkaline Phosphatase (AP) reaction buffer. When a coloured signal was achieved, the developing reaction was stopped by adding 20 ml of AP stop solution. Membrane was washed twice with deionised water and air dried at RT.

Quantification of protein expression and data analysis

The films from ECL detection or the membranes developed with NBT/BCIP were scanned using a GS-710 calibrated imaging densitometer (BioRad) and the intensity of the bands was quantified. Data are expressed as mean ± SEM of triplicate determinations from at least three independent experiments. Statistical significance analysis was conducted by one way analysis of variance (ANOVA). Unless otherwise noted, a level of statistical significance is considered * $p < 0.05$ versus control.

ASSAY OF PROTEIN PHOSPHATASES

Preparation of [γ-^{32}P]ATP

Fifty µl of [γ-^{32}P]ATP (250µCi - 3000 Ci/mmol, Amersham Pharmacia) were transferred to a 1.5 ml screw cap microcentrifuge tube, shielded in a Plexiglas box. The original vial was washed out with part of 445 µl of water and the washings transferred to the [γ-^{32}P]ATP tube. The remainder of water plus 5 µl of 100 mM ATP (pH was adjusted to 7.0 with NaOH and aliquots were kept at -20 ºC; 1/1000 dilution should have A_{260} of 1.54) was added to the container and mixed by pipetting up and down. Duplicates of 10 µl aliquots of 1/100 dilution in water were counted in a scintillation counter. Dilutions up to 1/1000 were also performed to measure A_{260} to check concentration and to determine specific activity.

Preparation of ^{32}P-labelled phosphorylase *a*

Phosphorylase kinase and phosphorylase *b* prepared solutions were incubated at 30 ºC for 10 min. The following components were mixed together, ATP was the last to be added:

Phosphorylase kinase	16 µl
100 mM Mg acetate	32 µl
100 mM $CaCl_2$	2 µl
Microcystin	3 µl
Phosphorylase *b*	200 µl
Buffer B	847 µl
[γ-^{32}P]ATP	500 µl
Total	1600 µl

Incubation took place for 2 h at 30 ºC. An equal volume of ice-cold 90% saturated ammonium sulphate was added. The mixture was left on ice for 30 min to precipitate protein, and centrifugated at 19000 g for 15 min at 4 ºC. The pellet was ressuspended gently in buffer C (not more than 5 ml per 100 mg of phosphorylase) using a pipette. An equal volume of ice-cold 90% saturated ammonium sulphate was added and the steps of precipitation on ice and centrifugation were repeated, to remove excess of ATP. The

suspension was dialysated for 24 h at 4 ºC against 4x 1 L of buffer D, to remove residual ATP. The dialysis buffer was monitored for radioactivity at each change. During dialysis the phosphorylase *a* crystallized forming a cream-coloured suspension. The contents of the dialysis bag were carefully empty into a microcentrifuge tube, left on ice for 2 h to ensure complete crystallization, centrifuged at 19000 g for 15 min at 4 ºC and the obtained crystals gently ressuspended in not more than 500 µl of buffer E. A sample was taken and diluted 100x in buffer E to determine the specific radioactivity. The concentrated solution was swirled before sampling to ensure complete ressuspension, and the diluted sample warmed at 30 ºC to dissolve the crystals. The A_{280} of 1/100 dilution was determined to estimate protein content (A_{280} is 1.31 for a 1 mg/ml solution), and triplicates of 10 µl aliquots were counted to determine the radioactivity. The specific radioactivity should correspond to 1 mole of phosphate per mole of 97.4 kDa subunit. Aliquots were pipetted into 1.5 ml microcentrifuge tubes and stored at 4 ºC (were not freeze as this denatures phosphorylase). The size of each aliquot varied depending on the exact protein concentration of the preparation, and was adjusted so the concentration of phosphorylase *a* was 3 mg/ml after the addition of 0.5 ml of buffer C and caffeine (see below).

Assay of phosphorylase phosphatase (PP1 and/or PP2A) activity

0.1 ml of caffeine and 0.4 ml of buffer C were added to one vial of [^{32}P]-phosphorylase *a* to give a 3 mg/ml (30 µmol/L) solution, and stored on ice. Occasionally, the redissolved phosphorylase *a* substrate showed slight cloudiness due to trace denaturation. When it occurred the vial was simply warmed for 1-2 min at 30 ºC and the precipitated spined out at 12000 g for 2 min and vial kept at RT (returned to storage at 4 ºC after being used). The protein phosphatase sample (crude extract or purified PP1 or PP2A) was diluted in buffer A. Several dilutions were checked to find the one that released 5-10% of the total counts. Microcentrifuge tubes (1.5 ml) were labelled, duplicates for each sample and two more for blanks. Blanks were included as the first and last tubes in every set of assays: in this way, any phosphatase carry-over due to contaminated pipette tip being accidentally inserted into the vial substrate was noticed. Using phosphorylase *a* as substrate, the blank should be <1% of the total counts: if the blank value rose above 5% of the total, the batch of substrate in use was discarded and a fresh aliquot was made. Into each sample tube, 10 µl of diluted protein phosphatase were pipetted and 10 µl of buffer A

(no enzyme) into blank tubes, and tubes were placed on ice. To each tube, 10 µl of buffer B (containing inhibitor/activator as required) was added, and tubes were placed on ice. Sample and blank tubes were removed from ice and placed in a water bath at 30 ºC. The phosphatase assay was started by adding 10 µl of [^{32}P]phosphorylase *a* at 10 or 15 sec intervals. The mix was vortexed and incubated at 30 ºC for 10 min. The reactions were stopped at 10 or 15 sec intervals by adding 100 µl of 20% (w/v) TCA to each tube, and the suspensions vortexed. Tubes were centrifuged at 12000 g for 2 min at RT in a microcentrifuge to sediment the precipitated protein. From each clear TCA supernatant, 100 µl were transferred to a second equally labelled set of tubes and the scintillations counted in a scintillation counter. A tube with 10 µl of [^{32}P]phosphorylase *a* (labelled "Total") was included. The calculation was made as follows: one unit (U) of protein phosphatase activity releases 1 µmol phosphatase from phosphorylase *a* per minute in a standard assay. Therefore the calculation for 10 min assay is:

c.p.m. released = Sample c.p.m. – Blank c.p.m.

$$\text{Activity (mU/ml)} = \frac{\text{c.p.m. released}}{\text{c.p.m. Total}} \times \frac{0.3}{10} \times \frac{130}{100}$$

0.3 is the number of nanomoles of phosphorylase in the assay, 10 is the incubation time in minutes, 100 is to convert the results for 1 ml rather than 10 µl of enzyme, and 130/100 is the fraction of the TCA supernatant that is counted.

SELDI-TOF MS ANALYSIS

Chip pretreatments

IMAC3 chip:

Draw an outline for each spot with a hydrophobic pen. Load 10 µl of 100 mM cupric sulfate ($CuSO_4$) solution and incubate in humid chamber for 15 min. Do not allow solution to dry. Rinse chip under running deionised water for 10 sec. Repeat loading cupric sulfate solution for another 10 min. Rinse chip under running deionised water for

10 sec. Equilibrate chip with 10 ml of the respective binding buffer for 10 min (in a 15 ml sterile tube) at RT with vigorous shaking. Transfer chip to bioprocessor.

H4 chip:

Pre-treat chip with 10 ml of 50% Acetonitrile (ACN) for 2 min. Remove ACN solution and add 10 ml of PBS for 2min. Equilibrate chip with 10 ml of the respective binding buffer for 10 min (in a 15 ml sterile tube) at RT with vigorous shaking. Transfer chip to bioprocessor.

NP20, SAX2 and WCX2 chips:

Equilibrate chip with 10 ml of the respective binding buffer for 10 min (in a 15 ml sterile tube) at RT with vigorous shaking. Transfer chip to bioprocessor.

Binding buffers

Each chip has appropriate binding buffers that are used to equilibrate the chips before being assembled into bioprocessor, to dilute samples and to perform the washes after incubation. After decide which chip and buffer are going to be used, always use the same buffer in each step.

IMAC chip: PBS/0.5 M NaCl

H4 chip: PBS/0.5 M NaCl
PBS/25% ACN

NP20 chip: 50 mM HEPES pH 7.4

SAX2 chip: 50mM Tris pH 9.0 (low stringency)
50mM Sodium acetate pH 4.5 (high stringency)

WCX2 chip: 50mM Tris pH 9.0 (high stringency)
50mM Sodium acetate pH 4.5 (low stringency)

Bioprocessor assembly

Bioprocessor consists on a metal device (the bracket) were chips (1 to12 chips) are assembled side by side and a plastic 96-well plate (the top) that is tightly assembled over the chips. A plastic gasket is assembled between the bracket and the top to avoid any leaks. When using less than 12 chips, dummy chips must be used to fill the empty slops in the 96-well bioprocessor. Do not use gloves or just use powder-free ones for handling chips and bioprocessor.

Equilibrate the chips with 10 ml of respective binding buffers (in 15 ml sterile tubes for 10 min at RT with vigorous shaking), assemble them in the bracket side by side and putting all with the same orientation (the chip has 8 spots identified from A to H, put A to the top, H to the bottom). Connect the gasket to the top and assemble them to the bracket with the chips. Close tightly. Load the samples avoiding touch the spot surface and surrounding coating of the chip. Incubate at RT for 90 min with vigorous shaking. Wash quickly each well with 100 µl of corresponding binding buffer. Remove the chips from the bioprocessor. Wash the chips with 10 ml of binding buffer 3 times for 5 min each in 15 ml conical centrifuge tubes with vigorous shaking. Rinse briefly with 10 ml of HPLC grade water and let to air dry. Load fresh EAM molecule solution (2 µl/spot) and allow to air dry for 10 min. Analyze chips by SELDI-TOF analysis using the ProteinChip System.

APPENDIX III – Technical information

CIPHERGEN PROTEINCHIP TECNOLOGY

ProteinChip Arrays

The ProteinChip Array consists of a metal base with 8 chemically active sites or "spots" where the actual sample binding occurs.

ProteinChip Arrays provide a variety of surface chemistries that allow researchers to optimize protein capture and analysis. The surface chemistries of the arrays include a series of classic chromatographic chemistries and specialized affinity capture surfaces. Classic chromatographic surfaces include normal phase for generic protein binding; hydrophobic surfaces for reversed-phase capture; cation and anion exchange surfaces; and immobilized metal affinity capture (IMAC) for metal-binding proteins. Specific proteins of interest can be covalently immobilized on pre-activated surface arrays, enabling customized experiments to investigate antibody-antigen, DNA-protein, receptor-ligand and other molecular interactions.

H4 Arrays

Hydrophobic surface arrays, are used for capturing proteins through hydrophobic interactions. The active spots chains of 16 methylene groups that can bind proteins through reverse phase chemistry via alanine, valine, leucine, isoleucine, phenylalanine, tryptophan, or tyrosine.

IMAC3 Arrays

Immobilized Metal Affinity Capture arrays, can be used to capture molecules that bind divalent cationic metals such as nickel, gallium, copper and zinc.

NP1 and NP2 Arrays

Normal phase arrays, are used for general binding of proteins for analysis. The chemistries on the active spots of both arrays contain silicon oxide which allows proteins to bind via serine, threonine or lysine. NP1 and NP2 arrays can be used to calibrate using peptides or proteins of known molecular weight.

PS1 and PS2 Arrays

Preactivated surface chip arrays, are used to covalently immobilize biomolecules for the subsequent capture or proteins from complex biological samples.

SAX2 Arrays

The Strong Anion Exchange arrays, can be used to analyze molecules with a negative charge on the surface. The active spots contain cationic, quaternary ammonium groups that interact with the negative charges on the surface of target proteins, e.g., aspartic acid or glutamic acid.

Uses for SAX2 arrays include: selective analysis of proteins with low pI's and for biomarker discovery.

WCX2 Arrays

The Weak Cation Exchange arrays, can be used to analyze molecules with a positive charge on the surface. The active spots contain weak anionic carboxylate groups that interact with the positive charges on the surface of the analyte, e.g., lysine, arginine or histidine.

Uses for WCX2 arrays include: selective analysis of proteins with low pI's and for biomarker discovery.

EAM molecule

EAM (Energy Absorbing Molecule) are the molecules that assist in the desorption and ionization of the analyte (protein or peptide). The EAM is applied in organic solvent, solubilizing many proteins on the chip surface. As the EAM solution dries, the proteins co-crystallize with the EAM. These crystals absorb the laser energy and generate the

ionized proteins detected by the ProteinChip Reader. In general, the EAM is chosen based on the molecular weight of the analyte of interest.

Isoelectric point

The isoelectric point of a protein (pI) is simply the pH value at which the protein has no net charge. The pI of a protein results from the charges of all the amino acid groups on the protein, plus the N-terminus and C-terminus. Acidic amino acid residues (e.g., aspartic acid, glutamic acid) decrease the pI of a protein. Basic amino acid residues (lysine, arginine, histidine) increase the pI of a protein.

The isoelectric point of a protein will strongly influence how well it binds to the ionic ProteinChip Arrays. Proteins with a low pI bind strongly to the anion exchange surface (SAX2). If the buffer solution is lowered below the pI of the protein, the protein will begin to bear a net positive charge and will bind more weakly to the surface of the array. Proteins with a high pI bind strongly to the cation exchange surface (WCX2). If the buffer solution is increased above the pI of the protein, the protein will begin to bear a net negative charge and will bind more weakly to the surface of the array.

The ProteinChip Reader (Series PBS II)

The ProteinChip Reader is a laser desertion/ionization time-of-flight mass spectrometer that uses state-of-the-art ion optic and laser optic technology. The laser optics maximize ion extraction efficiency over the greatest possible sample area, and thus increase analytical sensitivity and reproducibility. The Reader's ion optics incorporate a four-stage, time-lag-focusing ion lens assembly that provides precise, accurate molecular weight determinations with excellent mass sensitivity.

The ProteinChip Software

Ciphergen's ProteinChip Software controls all aspects of the ProteinChip Reader and facilitates data collection and analysis.