Universidade de Aveiro Departamento de Biologia
2006

Ana Sofia Direito dos Santos Duarte

Cardosinas no estabelecimento de culturas primárias de neurónios

Universidade de Aveiro Departamento de Biologia
2006

Ana Sofia Direito dos Santos Duarte

Cardosinas no estabelecimento de culturas primárias de neurónios

tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Bioquímica, realizada sob a orientação científica da Professora Doutora Marlene M. T. Barros, Professor Auxiliar da Universidade de Católica Portuguesa e do Professor Doutor Euclides Pires, Professor Associado no Departamento de Bioquímica da Universidade de Coimbra.

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio.

To my family, for their unconditional love, support, patience, and for giving me the inspiration and strength to reach my goals.

o júri

presidente

Prof. Dr. Joaquim José Borges Gouveia
professor catedrático do Departamento de Economia, Gestão e Engenharia Industrial da Universidade de Aveiro

Prof. Drª. Marlene Maria Tourais de Barros
professora auxiliar da Universidade Católica Portuguesa

Prof. Dr. Euclides Manuel Vieira Pires
professor associado da Faculdade Bioquímica da Universidade de Coimbra

Prof. Drª. Emíllia Conceição Pedrosa Duarte
professora auxiliar da Faculdade de Zoologia da Universidade de Coimbra

Prof. Dr. Carlos José Fialho da Costa Faro
professor auxiliar da Faculdade de Bioquímica da Universidade de Coimbra

Prof. Dr. Edgar Figueiredo da Cruz e Silva
professor associado do Departamento de Biologia da Universidade de Aveiro

Prof. Drª. Maria de Lourdes Gomes Pereira
professora associada c/ agregação do Departamento de Biologia da Universidade de Aveiro

Prof. Dr. Amadeu Mortágua Velho da Maia Soares
professor catedrático do Departamento de Biologia da Universidade de Aveiro

agradecimentos

I take this opportunity to some special people, without whom I would not have been able to succeed with my work. Most important, I would not be the person I am today!

To my principal supervisor Prof. Marlene Barros for your trust and for your encouragement through this combined field of biochemistry-biomedicine. You really give the kind of support that makes people grow!
My second supervisor Prof. Euclides Pires: Thanks for all your support and extraordinary scientific skills that always help and guide me in good and bad times.
Prof. Emília Duarte: even thought you never have been my "official" supervisor, you have without exception shown that you trusted me. You have given me the practical chance to develop this work, providing me with excellent research facilities in which to carry out this research at Center for Neuroscience and Cell Biology of University of Coimbra.
To Prof. António Correia for your friendship and support, otherwise, where would we be now without you? Thank you to give me the opportunity of carrying out this work among my true friends.
Fátima, my deepest thanks for all of the work that you have performed during these years, mainly by supporting me in the most difficult part of this work (animal handling including euthanasia). You are an excellent person.
I wish to express my honest gratitude to Ana, Inês, Joana, Raquel, Rita, Cristina, Marco, Tiago … all team in the laboratory … not only for sharing all stock solutions with me but especially for creating a nice working environment. Their fellowship has been invaluable to me for adjusting to a new working place.
I am sincerely grateful to Rui Oliveira and Isaura Simões for helping me to understand the "world" of RNA, thank you for technical support and for your enlightening discussions.
I would like to thank Prof. Adelaide Almeida and Prof. Angela Cunha for providing me the opportunity to analyse some of my immunocytochemical preparations by fluorescence microscopy. I would also like to acknowledge Prof. Lourdes Pereira who did not directly participate in the present work, but always make me feel her support. Thanks to Ricardo, Helena and "Sr. Aldiro" for their energy and comic relief when things started to get tricky.
Additionally, I want to extend my acknowledgments to the Department of Biology and to Centre of Cell Biology of University of Aveiro that provide the facilities that allow the success of this work.
All my friends deserve my special thanks for bringing joy into my life. I warmly thank Cristina, Cláudia, Anabela, Nuno, Isabel, Joca, Fátima, Marta, Maria João, Daniela and, my eternal treasure, Alexandra. It is privilege to me to share such pleasant moments with you (inside or outside the lab).
My heartfelt thanks to Prof. Arthur Moir for his careful review of my manuscripts and for his precious comments on my work, but mostly I want to express my appreciation for his never-ending support, friendship and encouragement throughout the years.
And a great thank to all of you not mentioned, but indeed have a huge place in my heart. I am sure that you know that I think of you with respect and love.
My deepest thanks go to my family especially to my parents, for their loving care and encouragement and for giving me an excellent foundation from which to build my life.
Last, but not least, I would like to dedicate this thesis to my husband Carlos and my wonderful daughter Rita for their unfailing love, patience, and understanding through it all. You give me strength to go further… I love you!

This work was supported by the Fundação para a Ciência e a Tecnologia, Portugal.

palavras-chave

cardosinas, dissociação de tecidos, culturas celulares, culturas neuronais, tripsina, metaloproteinses da matriz

resumo

O estabelecimento de culturas celulares primárias constitui um aspecto relevante para o estudo da biologia celular e molecular. Várias linhas celulares humanas estão disponíveis contudo, muitas adquiriram alterações genéticas significativas, incluindo a delecção de importantes genes reguladores. Neste sentido, é importante a criação de novos métodos que permitam aceder ao natural comportamento celular. Para analisar as actividades celulares relacionadas com a expressão de proteínas extracelulares da matriz, determinantes no processo de ramificação das neurites, procedeu-se ao desenvolvimento de um novo protocolo para isolamento e estabelecimento de culturas neuronais embrionárias de rato utilizando cardosinas.

As cardosinas são proteases aspárticas de plantas, extraídas dos pistilos de *Cynara cardunculus* L., tendo sido usadas neste trabalho como uma nova ferramenta para desagregação de tecidos. As cardosinas foram comparadas com a tripsina, em termos de efeito durante o processo de isolamento de neurónios corticais. Conjuntamente, os resultados morfológicos e funcionais sugerem uma potencial aplicação das cardosinas em técnicas de isolamento de diferentes tipos celulares. Os resultados apresentam vantagens claras, em termos da viabilidade celular, do rendimento, sugerindo a implementação das cardosinas para isolamento celular, em alternativa à tripsina.

Os neurónios corticais resultantes atingiram rácios de expressão MMPs/TIMPs após 24 horas em cultura proporcionais ao desenvolvimento e proliferação das neurites. A célere recuperação celular do processo de isolamento está relacionada com uma especificidade mais restrita das cardosinas o que suporta a sua aplicação, não só para o estabelecimento de culturas primárias, mas também para os procedimentos de subcultivo. Esta recuperação mais rápida sugere que, durante a desagregação do tecido nervoso, as cardosinas seguem um mecanismo menos agressivo para os neurónios que a tripsina. Este aspecto pode ter particular interesse em procedimentos de autotransplante, uma vez que a manipulação das culturas celulares deve preservar a integridade celular, para uma consequente reabilitação mais rápida do paciente.

keywords cardosins, tissue dissociation, cell culture, neuronal cultures, trypsin, matrix metalloproteinases

abstract

The establishment of primary cell cultures is invaluable for studying cell and molecular biological questions. A variety of human cell lines exist, however, most have acquired significant genetic alterations from their cells of origin, including deletion of important regulatory genes. Therefore is important to create new methods to ascertain natural cell behaviour. In order to analyze cellular activities related to extracellular matrix protein expression, determinant for neurite pathfinding, a new protocol was established for isolating and culturing neuronal cells from embryonic rats by using cardosins.
Cardosins are plant aspartic proteinases extracted from the pistils of *Cynara cardunculus* L. and were used in the present work as novel tool for tissue disaggregation. Cardosins were compared to trypsin effect in the isolation of cortical neurons. Both morphological and functional data suggest a potential application of cardosins in isolation techniques for different cellular types. The results demonstrate clear advantages, in terms of improved cell viability, increased yields, suggesting cardosins implementation as alternative to use trypsin for cell isolation.
The resultant cortical neurons revealed to achieve MMPs/TIMPs expression ratio after the first 24 hours in culture matching with a superior neurite outgrowth and dendritic extension. This early cell recovery to isolation procedure is related to the narrow specificity of cardosins that support their application for establishment of primary cell culture and for subcultivation procedures. The increase on neuronal recover after cell plating suggesting that, during brain disaggregation, cardosins follow a mechanism that is less aggressive to neurons than trypsin. This could be particularly interesting for autotransplantation procedures, as cell culture manipulation would preserve cell integrity, leading to a faster patient rehabilitation.

ESTABLISHMENT OF PRIMARY NEURONAL CULTURES USING CARDOSINS

∴ Effect on MMPs and TIMPs expression on neuronal regrowth ∴

Thesis submitted for fulfilment of PhD Degree in Biochemistry

by

ANA SOFIA DUARTE

CENTRE FOR CELL BIOLOGY, UNIVERSITY OF AVEIRO

AVEIRO 2006

The brain is the only organ that studies itself and Neuroscience is a multidisciplinary study of the brain and its performance. It is a new discipline that has grown out of modern needs to solve practical medical problems as well as address the continuing human desire to discover more about ourselves.

The ancestral Alkmaion of Croton (500 - 450 BC, Greece) was the first to place sensation in the brain and claimed that there was a conduct to transmit the sensory impressions that external objects made on the eyes - and he concluded that the brain was the seat of the intellect, and thus was one of the first cephalocentric protagonists.

Presently, neuroscientists are interested in questions such as: What is mind? Why do we need to sleep? Why do people feel emotions? What causes Alzheimer's Disease? What happens in the brain when we learn? And how does brain develop before birth? Neuroscience is nowadays a combined field that integrates Psychology, Biology, Chemistry, and several other traditional disciplines in the study of brain structure, physiology and behaviour including human emotional and cognitive functions.

TABLE CONTENTS Page

ABBREVIATIONS

BICINE	N,N-bis-(2-hydroxyethyl)glycine
cDNA	DNA Complementary To RNA
CNS	Central Nervous System
DNA	Deoxyribonucleic Acid
DAN	Diazoacetil Norleucine Methyl Ester
DG	Dentate Gyrus
DMSO	Dimethyl sulphoxide
dNTP	Deoxynucleotide Triphosphate
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
ECS	Electroconvulsive Seizures
EDTA	Ethylenediaminetetraacetic Acid
EGL	External Granular Layer
EGTA	Ethyleneglycol-Bis (Beta-Aminoethyl Ether)- N, N, N',N'-Tetraacetic Acid
ER	Endoplasmic Reticulum
KA	Kainic Acid (Kainite)
LRP	Low-Density Lipoprotein Receptor-Related Protein
MMP	Matrix Metalloproteinase
mRNA	messenger RNA
MT-MMP	Membrane-Type Matrix Metalloproteinase
NGF	Nerve Growth Factor
NO	Nitric Oxide
PAGE	Polyacrylamide Gel Electrophoresis
PAI	Plasminogen Activator Inhibitor
PBS	Phosphate-Buffered Saline

PCR	Polymerase Chain Reaction
PGs	Proteoglycans
PMSF	Phenylmethylsulfonyl Fluoride
PSI	Plant Specific Insert
RNA	Ribonucleic Acid
RNase	ribonuclease
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
TBS-T	Tris-Buffered Saline-Tween
TEMED	N,N,N',N'-tetrametiletilenodiamine
TNF	Tumor Necrosis Factor
TIMP	Tissue Inhibitor Of Matrix Metalloproteinases
tPA	Tissue Plasminogen Activator
TRIS	2-Amino-2-hydroxymethylpropane-1,3-diol
uPA	Urokinase Plasminogen Activator
uPAR	Upa Receptor
UV	Ultraviolet

LIST OF FIGURES

1. Introduction

3. Establishment of neuronal primary cultures

4. MMPs involvement in the mechanisms of neuronal regeneration and functional recovery

7. Appendix

1. INTRODUÇÃO

1. Introduction

Research using human and animal cell preparations continues to be an extremely active area of investigation. In fact, it has become very beneficial for diverse applications in biotechnology and biomedical research. Originally used as substrates for the production of viral vaccines, cell cultures became an indispensable tool to produce a variety of products, including biopharmaceuticals, monoclonal antibodies and products for gene therapy.

The use of cell cultures constitutes also adequate test systems for studying biochemical pathways, pathological mechanisms or intra- and intercellular responses. Human cell culture, stem cell culture in particular, is developing new research tools, new knowledge about pathways of cell differentiation and opening new perspectives of cell transplantation therapy for human diseases.

For instance, transplantation of embryonic dopaminergic neurons has been performed in animals and in patients with Parkinson's disease for several years[1,2,3]. However, the availability of human embryonic donor tissue for clinical transplantations is limited and only 5-10% of the grafted neurons survive[4].

To overcome these limitations there is a need to develop strategies to improve cell survival. Not only for transplantation purposes but for general applications of cell cultures where it is crucial to preserve cell integrity and its native features. The establishment of primary cell cultures is invaluable for studying cell and molecular biological issues. A variety of human cell lines exist, however, most have acquired significant genetic alterations from their cells of origin, including deletion of important regulatory genes. Therefore is important to create new methods to ascertain natural cell behaviour.

To achieve maximal neuroprotection is important to be aware to the steps of cell preparation procedures when cell death is pronounced.

---

[1] Hagell P, Brundin P (2001). Cell survival and clinical outcome following intrastriatal transplantation in Parkinson disease. J Neuropathol Exp Neurol. 60(8): 741-52. Review.

[2] Lindvall O, Hagell P. Clinical observations after neural transplantation in Parkinson's disease, in: S.B. Dunnett, A. Bjo¨rklund (Eds.), Progress in Brain Research, Vol. 127, Elsevier Science, 2000, pp. 299-320.

[3] Karlsson J, Emgard M, Brundin P. (2002). Comparison between survival of lazaroid-treated embryonic nigral neurons in cell suspensions, cultures and transplants. Brain Res. 955(1-2): 268-80.

[4] Kordower JH, Freeman TB, Chen EY, Mufson EJ, Sanberg PR, Hauser RA, Snow B, Olanow CW (1998). Fetal nigral grafts survive and mediate clinical benefit in a patient with Parkinson's disease, Mov. Disord. 13 383–393.

The most important stage that involves cell death is during brain dissection and enzymatic dissagregation for cell dissociation. During this phase, characterised by enzymatic cleavage of extracellular matrix (ECM) components or membrane-associated proteins, neurons are axotomized and cell–cell contact is disrupted which may lead to apoptosis compromising cell isolation efficiency[3,5].

The ECM molecules that are particularly implicated in the major steps of nervous system development are the basal membrane components; however, it is not easy to understand the exact roles of each molecule because of their complexity and redundancy. Interactions between cells and their surrounding ECM are required to generate the specific features of tissue architecture.

During neuronal development, the growth cone, the leading structure of growing axons, senses guidance cues from the surrounding environment and implements directed outgrowth. Axonal regeneration requires extensive growth cone motility and infiltration within damaged and degenerating nervous tissue. Substantial evidence now indicates that neurons secrete matrix-degrading enzymes and actively remodel surrounding ECM substrata of the mammalian nervous system[6,7,8]. Axon outgrowth can thus perform as a concept for tissue invasion in tumorigenesis. Neuronal cells growth cone "sniffs out" the extracellular environment for signals that instruct the axon which way to grow. These signals, called guidance cues, can be fixed in place or diffusible; they can attract or repel axons. It has long been conjured that proteolytic activity on neuronal growth cones controls their migratory activity[9]. Matrix metalloproteinases (MMP) form one of the most important families of proteinases that participate in the processing of bioactive molecules. It is believed that the main function of proteases is to create penetrable paths for axon extension and to modulate the activities of receptors and ligands through proteolytic processing. Both roles have been clearly demonstrated in recent studies. Plasminogen activators and MMPs are expressed by neurons and

[5] Li AE, Ito H, Rovira I, Kim K, Takeda K, Yu ZY, Ferrans VJ, Finkel T (1999). A role for reactive oxygen species in endothelial cell anoikis. Circ. Res. 85: 304–310.

[6] Monard D (1988) Cell-derived proteases and protease inhibitors as regulators of neurite outgrowth. Trends Neurosci 11:541-544.

[7] Pittman RN, Buettner HM (1989) Degradation of extracellular matrix by neuronal proteases. Dev Neurosci 11:361-375.

[8] Fambrough D, Pan D, Rubin GM, Goodman CS (1996) The cell surface metalloprotease/disintegrin Kuzbanian is required for axonal extension in *Drosophila*. Proc Natl Acad Sci USA 93:13233-13238.

[9] Krystosek, A. and Seeds, N.W. (1981) Plasminogen activator release at the neuronal growth cone. Science 213, 1532–1534.

released by growth cones, implicating the growing tip of axons in the proteolysis of ECM substrata[10].

Hence, MMPs relevance in neuronal growth and regeneration constitutes another important remark of the present study. The matrix metalloproteinases have been viewed as bulldozers[11], destroying the extracellular matrix to permit normal remodelling and contribute to pathological tissue destruction and tumour cell invasion. More recently, the identification of specific matrix and non-matrix substrates for MMPs and the elucidation of the biological consequence of cleavage indicate that we need to expand upon the simplistic view that MMPs punch holes in basement membrane and extracellular matrix structures to a much more sophisticated vision of specific proteolytic events.

MMPs can regulate the bioavailability and/or activity of growth factors by cleavage of both matrix and non-matrix substrates or by mediating receptor turnover, and their activity is independent of the tissue source.

There are some general examples of MMPs that directly cleave and activate growth factors. MMP-2 and MMP-9 directly process TGF-β into an active ligand[12]. Proteolytic processing of interleukin 1-β (IL1-β) from its precursor form to an active form has been reported to be carried out by MMP-2, MMP-3 and MMP-9[13].

Cell migration is a complex process that requires the coordinated regulation of cell–cell attachments, cell–matrix attachment and matrix remodelling. It seems intuitive that matrix proteolysis could directly modulate cell–matrix adhesion either by removal of sites of adhesion or by exposing a binding site. This can translate into an effect on cell migration, as MMP-2-dependent cleavage of laminin-5 induces keratinocyte migration[14] and MT1-MMP cleavage of laminin-5 allows migration of a variety of cell types[15].

---

[10] Teesalu T, Kulla A, Asser T, Koskiniemi M, Vaheri A (2002). Tissue plasminogen activator as a key effector in neurobiology and neuropathology. Biochem Soc Trans 30(2):183-9.

[11] McCawley LJ, Matrisian LM (2001). Matrix metalloproteinases: they're not just for matrix anymore! Curr Opin Cell Biol.13(5):534-40. Review.

[12] Yu Q, Stamenkovic I (2000). Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-β and promotes tumor invasion and angiogenesis. Genes Dev 14: 163-176.

[13] Schonbeck U, Mach F, Libby P (1998). Generation of biologically active IL-1β by matri mxetalloproteinases: a novel caspase-1-independent pathway of IL-1β processing. J Immunol, 161:3340-3346.

[14]Giannelli G, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG, Quaranta V (1997). Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. Science, 277: 225-228.

[15] Koshikawa N, Giannelli G, Cirulli V, Miyazaki K, Quaranta V (2000). Role of cell surface metalloprotease MT1-MMP in epithelial cell migration over laminin-5. J Cell Biol, 148:615-624.

MMP activity may also modulate attachment and migration by cleaving cell-cell or cell-matrix receptors directly. For example, β4 integrin is cleaved by MMP-7[16], and MMP mediated proteolytic removal of E-cadherin has been demonstrated[17].

Cell migration is often initiated in response to a chemotactic stimulus. MMPs generate a chemotactic signal in several systems. MMP-9 is required for the release of vascular endothelial growth factor (VEGF) during long bone development, VEGF then acts as a chemoattractant for osteoblast recruitment[18]. MMPs also play a role in balance the chemotactic response. For example, monocyte chemoattractant protein (MCP)-3 is cleaved and inactivated by MMP-2, attenuating chemotaxis and the inflammatory response[19].

Perhaps the most significant consequence of the overexpression of metalloproteinases in the central nervous system (CNS) is the regulation of cell survival and death. Various mechanisms contribute to metalloproteinase-mediated cell death. The adherence of cells to the ECM provides survival signals through mechanisms that include the activation of integrin receptors that have engaged particular ECM proteins. When such anchored cells are detached from the substratum, the loss of integrin signalling can result in apoptosis, a phenomenon that has been referred to as anoikis.

Therefore, in the CNS, the degradation of ECM proteins and loss of integrin signalling by abnormally expressed MMPs can affect cell survival.

As pointed out before, in the nervous system there is substantial evidence that MMPs regulate axonal growth. The metalloproteinase activity is localized to the growth cones of neurons, and inhibition of metalloproteinase activity reduces growth cone motility[20].

---

[16] Von Bredow DC, Nagle RB, Bowden GT, Cress AE (1997): Cleavage of β4 integrin by matrilysin. Exp Cell Res 236: 341-345.

[17] Noe V, Fingleton B, Jacobs K, Crawford HC, Vermeulen S, Steelant W, Bruyneel E, Matrisian LM, Mareel M (2001). Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. J Cell Sci, 114:111-118.

[18] Engsig MT, Chen QJ, Vu TH, Pedersen AC, Therkidsen B, Lund LR, Henriksen K, Lenhard T, Foged NT, Werb Z, Delaisse JM (2000). Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing long bones. J Cell Biol, 151:879-890.

[19] McQuibban GA, Gong JH, Tam EM, McCulloch CA, Clark-Lewis I, Overall CM (2000). Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. Science, 289: 1202-1206.

[20] Sheffield JB, Krasnopolsky V, Dehlinger E (1994). Inhibition of retinal growth cone activity by specific metalloproteinase inhibitors in vitro. Dev. Dyn. 200, 79–88.

Additionally, growth factors and ECM molecules that promote neurite outgrowth, such as nerve growth factor (NGF) and laminin, have been correlated to an increase the expression of MMP-2 in neurons[21]. Several studies have demonstrated that MMPs have roles in axonal elongation[21] and are also implicated in their guidance[22] during normal development. Following injury there is no evidence that metalloproteinases facilitate regeneration of axons *in vivo*, but various lines of facts point towards this potential. One example is given by expression of metalloproteinases has been shown to correspond with periods of recovery. In regenerating sciatic nerve fibres, MMP-9 expression is co-localized with phosphorylated neurofilament M, a marker for regenerative elongation; the phosphorylated neurofilament M is also induced by MMP-9 treatment and inhibited by an anti-MMP-9 antibody treatment[23,24].
Overall, these studies emphasize that when MMPs are expressed in a spatially and temporally restricted manner during development or after an insult, they can have important roles.

Indeed, MMPs can perform either beneficial or detrimental activities in the CNS. Although these findings show that the net effect of the acute upregulation of many metalloproteinases in nervous system pathology is detrimental, certain MMPs that are discreetly expressed at specific sites, particularly during the repair or recovery phase, can have beneficial activity. For example, MMP-9 is detrimental in multiple sclerosis and in acute insults to the nervous system, such as stroke and spinal cord injury. However, MMP-9 has a beneficial effect on repair processes such as axonal regeneration and remyelination.
At least three factors might determine whether metalloproteinases are beneficial or detrimental after nervous system injuries: the stage of CNS injury, the type of injury inflicted, and the pathophysiology of the disorder involved.
Metalloproteinases are implicated in diseases of the nervous system, and, therapeutically, it is important to target their activity. Although the long-term

[21] Weeks BS, Nomizu M, Ramchandran RS, Yamada Y, Kleinman HK (1998). Laminin-1 and the RKRLQVQLSIRT laminin-1 a1 globular domain peptide stimulate matrix metalloproteinase secretion by PC12 cells. Exp. Cell Res. 243: 375–382.
[22] Webber CA, Hocking JC, Yong VW, Stange CL, McFarlane S (2002). Metalloproteases and guidance of retinal axons in the developing visual system. J. Neurosci. 22: 8091–8100.
[23] Shubayev VI, Myers RR (2004). Matrix metalloproteinase-9 promotes nerve growth factor-induced neurite elongation but not new sprout formation in vitro. J. Neurosci. Res. 77: 229–239.
[24] Demestre M, Wells GM, Miller KM, Smith KJ, Hughes RA, Gearing AJ, Gregson NA. (2004). Characterisation of matrix metalloproteinases and the effects of a broad-spectrum inhibitor (BB-1101) in peripheral nerve regeneration. Neuroscience 124, 767–779.

impact of metalloproteinase inhibitors in neurological conditions is not clear because of the complexity of metalloproteinase function, the evidence suggests that the short-term use of inhibitors after acute insults such as stroke and spinal cord injury will have beneficial outcomes.

In addition to MMPs, TIMPs (tissue inhibitors of metalloproteinases) expression has been implicated in several inflammatory disease of CNS. For example, the idea that TIMP-1 up-regulation modulates the general neuronal responses following excitotoxic injury has received increased attention in the recent literature. Tan and co-workers (2003)[25] showed that application of TIMP-1 protected neurons against excitotoxic injury induced with glutamate. Nevertheless, *in vitro* studies with primary human fetal astrocytes and further biological conformation using cerebrospinal fluid and brain tissue reflected differential TIMP-1 regulation. TIMP-1 mRNA and protein increased significantly with acute activation of astrocytes with IL-1β; however, cerebrospinal fluid and brain tissue from human immunodeficiency virus (HIV)-1-associated dementia patients showed significant down-regulation[26]. The reduction in protein expression ultimately brings about withdrawal of TIMP-1 neuroprotection and enhanced neuronal malfunction. These studies demonstrate that astrocytes may behave differently with acute versus chronic activation.

Gardner & Ghorpade (2003)[27] proposed a model for astrocyte TIMP-1 regulation in acute and chronic inflammation. Acute activation of neural cells (astrocytes, microglia, and neurons) by proinflammatory cytokines such as IL-1β may lead to enhanced levels of TIMP-1 in the tissue microenvironment and elicit a typical repair response early in injury (Fig. 1.1 A). In contrast, under sustained inflammatory conditions, such as those observed in chronic neurodegenerative diseases (human immunodeficiency virus (HIV)-1-associated dementia and Alzheimer's disease), TIMP-1 levels in the CNS decline significantly below homeostatic levels (Fig. 1.1 B).

[25] Tan HK, Heywood D, Ralph GS, Bienemann A, Baker AH, Uney JB (2003). Tissue inhibitor of metalloproteinase 1 inhibits excitotoxic cell death in neurons. Mol Cell Neurosci 22: 98 –106.

[26] Suryadevara R, Holter S, Borgmann K, Persidsky R, Labenze-Zink C, Persidsky Y, Gendelman HE, Wu L, Ghorpade A (2003). Regulation of tissue inibitor of metalloproteinase-1 by astrocytes: links to HIV-1 dementia. Glia 44: 47–56.

[27] Gardner J, Ghorpade A (2003). Tissue Inhibitor of Metalloproteinase (TIMP)-1: The TIMPed Balance of Matrix Metalloproteinases in the Central Nervous System. Journal of Neuroscience Research 74:801–806.

The illustration for these events (Fig. 1.1) shows the complexity of events involving TIMP-1 expression. A better understanding of the mechanism of this duality will lead to insights into development of new therapeutic options relevant to treatment of a variety of diseases involving neural injury.

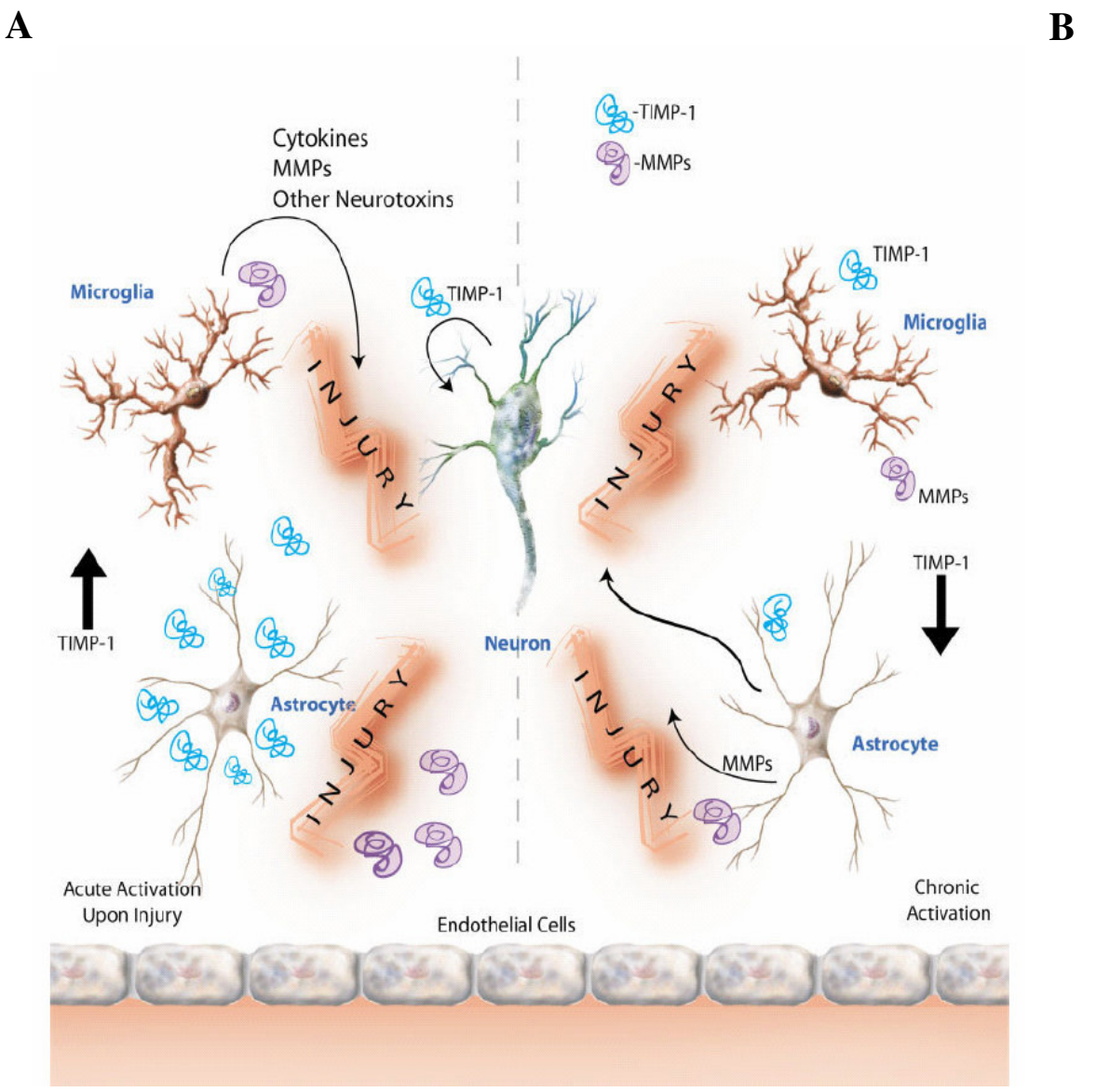


Figure 1.1 – Model for neuronal cells (astrocytes, microglia, and neurons) TIMP-1 regulation in acute (**A**) and chronic (**B**) inflammation proposed by Gardner & Ghorpade (2003).

The study of metalloproteinase and their endogenous specific inhibitors functions in the nervous system should open up new vision in CNS physiology and pathology. Actually, taking advantages of the increase on MMPs findings, these enzymes could be used as valuable tool to forecast cell behaviour and cell functional state. The broadly MMPs involvement in cell regulation mechanisms can be particularly helpful, to investigate, for instance, the effect of pharmaceutical drugs to be used in the treatment of several diseases, where MMPs might perform in an injurious manner.

To highlight the link between ECM processing and neuronal development, the following section provides an overview of some aspects of the biological role of MMPs and their specific TIMPs in the nervous system.

1.1 Matrix metalloproteinases and their endogenous inhibitors in the brain physiology: Brief review

Extracellular matrix is the glue that maintains tissue integrity, and consequently the balance between its production and degrading is of vital importance. Cell-cell and cell-ECM interactions provide essential information for controlling tissue events.

Matrix metalloproteinases (MMPs), an extracellular zinc-binding endopeptidase family, are instrumental in coordinating these interactions through the cleavage of ECM proteins and receptors[28,29] and regulate extracellular signalling pathways by selectively exposing ECM cryptic sites and inducing proteolysis of non-ECM receptors and ligands[30,31,32]. These processes play a pivotal role during tissue growth and morphogenesis, such as during trophoblast implantation, and mammary and bone development[33,3].

[28] Werb Z (1997). ECM and cell surface proteolysis: regulating cellular ecology. Cell 91: 439–442.
[29] Nagase H, Woessner JF Jr (1999). Matrix metalloproteinases. J. Biol. Chem. 274: 21491–21494.
[30] McCawley LJ, Matrisian LM (2001). Matrix metalloproteinases: they're not just for matrix anymore. Curr. Opin. Cell Biol. 13: 534–540.
[31] Galko MJ Tessier-Lavigne M (2000). Function of an axonal chemoattractant modulated by metalloprotease activity. Science 289: 1365–1367.
[32] Hattori M, Osterfield M Flanagan JG (2000). Regulated cleavage of a contact-mediated axon repellent. Science 289: 1360–1365.
[33] Vu TH Werb Z (2000). Matrix metalloproteinases: effectors of development and normal physiology. Genes Dev. 14: 2123–2133.

The first MMP activity described belongs to a collagenase found in the tail of a tadpole undergoing metamorphosis[34]. To date, 25 different vertebrate MMPs have been identified, of which 24 are found in humans.

They are designated by MMP1-28, however the names MMP4, MMP5 and MMP–6 are no longer used, as the originally socalled members were subsequently found to be MMP2 or MMP3[35]. The sequence homology with collagenase 1 (MMP-1), the cysteine switch motif PRCGXPD in the propeptide that maintains MMPs in their zymogen form (proMMP), and the zinc-binding motif HEXGHXXGXXH in the catalytic domain are the signatures used to assign proteinases to this family. The exception is MMP-23, which lacks the cysteine switch motif. MMPs generally consist of a prodomain, a catalytic domain, a hinge region, and a hemopexin domain, as it is represented in Fig. 1.2.

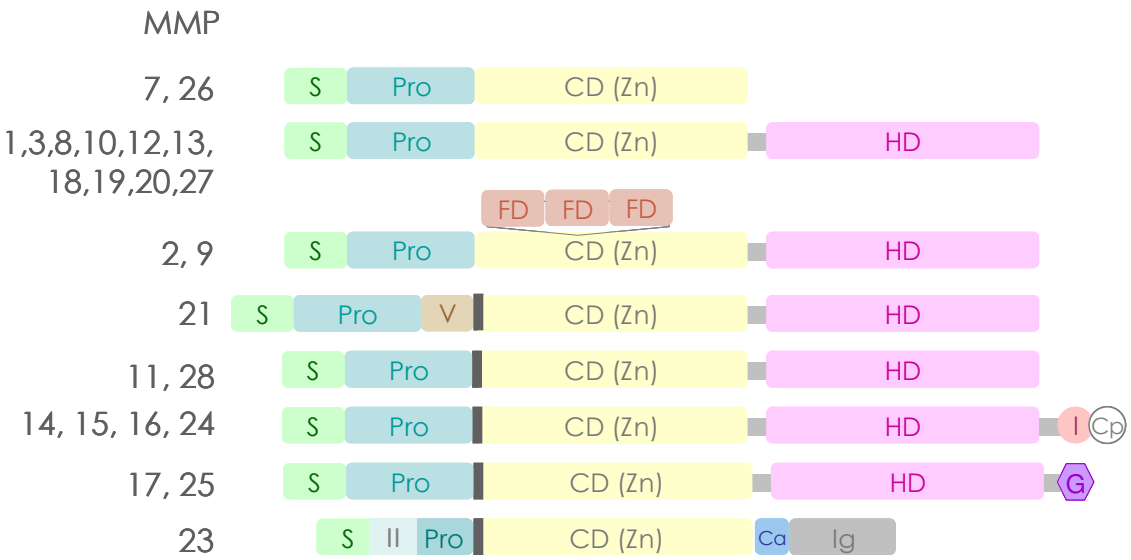


Figure 1.2 – Domain structure of matrix etalloproteinases (MMPs). The domain organization of MMPs is as indicated: S, signal peptide; Pro, propeptide; CD, catalytic domain; Zn, active-site zinc; HD, hemopexin domain; FD, fibronectin domain; V, vitronectin insert; I, type I transmembrane domain; II, type II transmembrane domain; G, GPI anchor; Cp, cytoplasmic domain; Ca, cysteine array region; and Ig, IgG-like domain. A furin cleavage site is depicted as a dark grey band between propeptide and catalytic domain (adapted from Visse & Nagase, 2003[36]).

[34] Gross J (1966). How tadpoles lose their tails. The journal of investigative dermatology. 47: 274-277.
[35] Yong VW (2005). Metalloproteinases: Mediators of Pathology and Regeneration in the CNS. Nat Rev Neurosci. 6: 931-944.
[36] Visse R, Nagase H. (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res. 92(8):827-839. Review.

The most widely studied disease that involves MMPs is cancer, mainly in terms of invasion and metastasis formation. In this case, the tumour cell is thought to use MMPs to overcome multiple structural barriers and establish a new focus of growth at a distant site from the primary tumour mass.

In the nervous system, MMPs have also been associated with pathogenesis, particularly in multiple sclerosis and malignant gliomas[37]. A growing literature has linked MMPs to stroke, to Alzheimer's disease and to viral infections of the CNS. As mentioned above, 24 mammalian MMPs and 4 of their endogenous tissue inhibitors of metalloproteinases, TIMPs, act together to control tightly temporally restricted, focal proteolysis of ECM (Table 1.1).

TIMPs are specific inhibitors that bind MMPs in a 1:1 stoichiometry. Four TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been identified in vertebrates[38] and their expression is regulated during development and tissue remodelling. TIMPs (21 to 29 kDa) have an N- and C-terminal domain of ~125 and 65 amino acids, respectively, with each containing three conserved disulfide bonds. The N-terminal domain folds as a separate unit and is capable of inhibiting MMPs[36]. TIMPs inhibit all MMPs tested so far, except that TIMP-1 fails to inhibit MT1-MMP[39]. Although each TIMP appears capable of inhibiting almost all MMPs, these proteins exhibit preferential inhibitory capacity. As the most described example, TIMP-1 and TIMP-2 can form a specific complex with the zymogens of MMP-9 and MMP-2, respectively, through the C-terminal domain of each molecule[40].

On the basis of substrate specificity, sequence similarity, and domain organization, vertebrate MMPs can be divided into six groups: Collagenases, Gelatinises, Stromelysins, Matrilysins, Membrane-Type MMPs (MT-MMPs) and other MMPs.

[37] Yong, VW, Krekoski, CA, Forsyth, PA, Bell, R, Edwards, DR (1998). Matrix metalloproteinases and diseases of the central nervous system. Trends Neurosci. 21: 75–80.
[38] Brew K, Dinakarpandian D, Nagase H (2000). Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophys Acta. 1477:267–283.
[39] Will H, Atkinson SJ, Butler GS, Smith B, Murphy G (1996). The soluble catalytic domain of membrane type 1 matrix metalloproteinase cleaves the propeptide of progelatinase A and initiates autoproteolytic activation: regulation by TIMP-2 and TIMP-3. J Biol Chem. 271: 17119–17123.
[40] Itoh Y, Ito A, Iwata K, Tanzawa K, Mori Y, Nagase H (1998). Plasma membrane-bound tissue inhibitor of metalloproteinases (TIMP)-2 specifically inhibits matrix metalloproteinase 2 (gelatinase A) activated on the cell surface. J Biol Chem. 273(38): 24360-7.

Table 1.1 - Matrix metalloproteinase family members

Member	Name	M latent/active (kDa)	Collagen substrates	Other ECM substrates
MMP1	Collagenase-1	55/45	I, II, III, VII, VIII, X	Agg, Gel, PG
MMP2	Gelatinase-A	72/66	I, III, IV, V, VII, X, XI, XIV	Agg, EL, FN, Gel, LN, PG, VN
MMP3	Stromelysin-1	57/45	III, IV, IX, X, XI	Agg, EL, FN, Gel, LN, PG, VN
MMP7	Matrilysin	28/19	IV, X	Agg, Casein, EL, FN, Gel, LN, PG, VN
MMP8	Collagenase 2	75/58	I, II, III, V, VII, VIII, X	Agg, EL, FN, Gel, LN
MMP9	Gelatinase B	92/86	IV, V, VII, X, XIV	Agg, EL, FN, Gel, PG, VN
MMP10	Stromelysin 2	57/44	III, IV, V, IX, X	Agg, EL, FN, Gel, LN, PG
MMP11	Stromelysin 3	51/44		
MMP12	Metalloelastase	54/45/22	IV	Casein, EL, FN, Gel, LN, PG, VN
MMP13	Collagenase 3	60/48	I, II, III, IV, VII, IX, X, XIV	Agg, FN, Gel
MMP14	MT1-MMP	66/56	I, II, III	Agg, EL, FN, Gel, LN
MMP15	MT2-MMP	72/60		Agg, FN, Gel, LN
MMP16	MT3-MMP	64/52	III	Gel, FN
MMP17	MT4-MMP	57/53		Fibrinogen/fibrin
MMP18	Collagenase 4	70/53	I	
MMP19	RAS I 1	54/45	IV	Gel, FN, LN
MMP20	Enamelysin	54/22		Amelogenin
MMP21	*Xenopus* MMP	70/53		
MMP22	Chick embryo MMP	51/42		Casein, Gel
MMP23	CA-MMP[41]			
MMP24	MT5-MMP			Gel
MMP25	MT6-MMP	34/28[42]	IV	Gel, FN
MMP26	Matrilysin 2/endometase	28/19		Gel
MMP27	Human MMP22			
MMP28	Epilysin	56/45		Casein

Agg (agrecan); Gel (gelatine); PG (proteoglicans); EL (elastin); FN (fibronectin); LN (laminin); VN (vitronectin)

Collagenases[43] (MMP-1, MMP-8, MMP-13 and MMP-18) that cleave interstitial collagens and other ECM and non-ECM molecules, **Gelatinases** (MMP-2 and MMP-9) that readily digest denatured collagen and contain three repeats of a

[41] Pei D. (1999) CA-MMP: a matrix metalloproteinase with a novel cysteine array, but without the classic cysteine switch(1). FEBS Lett 457:262-70.
[42] Duanqing P (1999) Leukolysin/MMP25/MT6-MMP: a novel matrix metalloproteinase specifically expressed in the leukocyte lineage Cell Research 9, 291-303.
[43] Lapiere ChM (2005). Tadpole collagenase, the single parent of such a large family. Biochimie. 87(3-4):243-7. Review.

type II fibronectin domain inserted in the catalytic domain, which bind to gelatin, collagens, and laminin[44], **Stromelysins**[45] (MMP-3 and MMP-10) that besides digesting ECM components, activate a number of proMMPs, **Matrilysins**[46] (MMP-7 and MMP-26) that processes cell surface molecules such as pro-α-defensin, Fas-ligand, pro–tumor necrosis factor (TNF)-α, and E-cadherin besides ECM components. **Membrane-Type MMPs** that, with the exception of MT4-MMP, are capable of activating proMMP-2 and can also digest ECM components and **other MMPs** (MMP-11, MMP-12, MMP-19, MMP-20, MMP-22, MMP-23, MMP-28) are examples of MMPs that are not classified in the above categories. MMP-11 is called stromelysin-3, but it is usually grouped with "other MMPs" because the sequence and substrate specificity diverge from those of MMP-3. MMPs are enzymatically activated by means of the cleavage of the propeptide[47]. Once activated, the MMPs are subject to inhibition by TIMPs.

Activation of proMMPs by plasmin is an important pathway *in vivo*. Plasmin is generated from plasminogen by tissue plasminogen activator (tPA) bound to fibrin and urokinase plasminogen activator (uPA) bound to a specific cell surface receptor. Plasmin has been reported to activate proMMP-1, proMMP-3, proMMP-7, proMMP-9, proMMP-10, and proMMP-13[48]. Activated MMPs can participate in processing other MMPs. The stepwise activation system may have evolved to accommodate advanced regulatory mechanisms to control destructive enzymes, considering that TIMPs may interfere with activation by interacting with the intermediate MMP before it is fully activated[36] (see Fig. 1.3). Nonetheless, MMPs activation can also occur either intracellularly by furin[49,50,,51,52] (including MMP-11, the six MT-MMPs, MMP-23, and epilysin) or on the cell surface (the most revised example is proMMP-2 activation).

---

[44] Allan JA, Docherty AJ, Barker PJ, Huskisson NS, Reynolds JJ, Murphy G (1995). Binding of gelatinases A and B to type-I collagen and other matrix components. Biochem J. 309:299–306.
[45] Suzuki K, Enghild JJ, Morodomi T, Salvesen G, Nagase H (1990). Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin). Biochemistry;29:10261–10270.
[46] Uria JA, López-Otín C (2000). Matrilysin-2, a new matrix metalloproteinase expressed in human tumors and showing the minimal domain organization required for secretion, latency, and activity. Cancer Res., 60:4745-4751.
[47] Leppert D, Lindberg RL, Kappos L, Leib SL (2001). Matrix metalloproteinases: multifunctional effectors of inflammation in multiple sclerosis and bacterial meningitis. Brain Res Brain Res Rev. 36(2-3):249-57. Review.
[48] Lijnen HR (2001). Plasmin and matrix metalloproteinases in vascular remodeling. Thromb Haemost. 86: 324-333.
[49] Pei D, Weiss SJ (1995). Furin-dependent intracellular activation of the human stromelysin 3 zymogen. Nature. 375: 244–247.
[50] Sternlicht MD, Werb Z (2001). How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol. 17: 463–516.

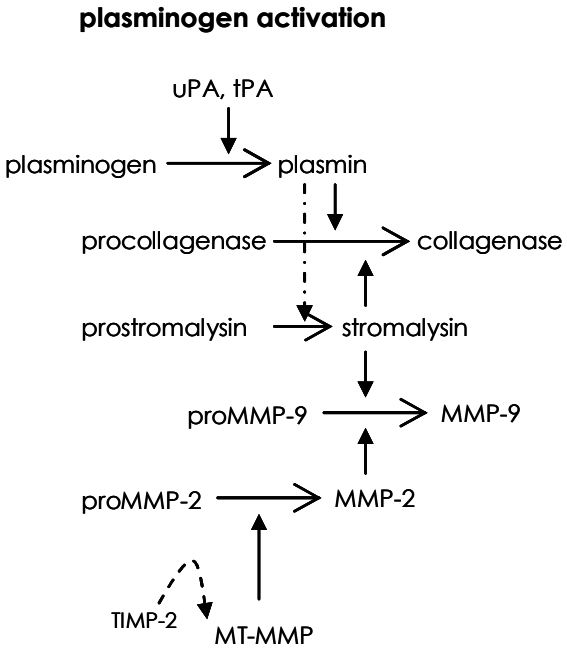


Figure 1.3 - The protease cascade for MMPs activation. uPA, urokinase plasminogen activator; tPA, tissue plasminogen activator (adapted from: Van den Steen *et al*., 2001[53]).

Most proMMPs are secreted from cells and activated in the extracellular space. MT1-MMP-mediated activation of proMMP-2 has been studied extensively. The unique feature of proMMP-2 activation is that TIMP-2 is required to assist it[54,55,56]. As represented in Fig. 1.4, proMMP-2 forms a tight complex with TIMP-2 through their C-terminal domains, therefore permitting the N-terminal inhibitory domain of TIMP-2 in the complex to bind to MT1-MMP on the cell surface.

The cell surface–bound proMMP-2 is then activated by an MT1-MMP that is free of TIMP-2. Otherwise, MT1-MMP linked to TIMP-2 can act as a "receptor" of proMMP-2. Effective activation of proMMP-2 is achieved when the ternary

[51] Marchenko GN, Strongin AY (2001). MMP-28, a new human matrix metalloproteinase with an unusual cysteine-switch sequence is widely expressed in tumors. Gene. 265: 87–93.
[52] Lohi J, Wilson CL, Roby JD, Parks WC (2001). Epilysin, a novel human matrix metalloproteinase (MMP-28) expressed in testis and keratinocytes and in response to injury. J Biol Chem. 276: 10134 –10144.
[53] Van den Steen P, Opdenakker G, Wormald M, Dwek R, Rudd P (2001). Matrix remodelling enzymes, the protease cascade and glycosylation. Biochimica et Biophysica Acta 1528: 61-73.
[54] Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI (1995). Mechanism of cell surface activation of 72-kDa type IV collagenase: isolation of the activated form of the membrane metalloprotease. J Biol Chem. 270: 5331–5338.
[11] Butler GS, Butler MJ, Atkinson SJ, Will H, Tamura T, van Westrum SS, Crabbe T, Clements J, d'Ortho MP, Murphy G (1998). The TIMP2 membrane type 1 metalloproteinase "receptor" regulates the concentration and efficient activation of progelatinase A: a kinetic study. J Biol Chem. 273: 871–880.
[56] Wang Z, Juttermann R, Soloway PD (2000). TIMP-2 is required for efficient activation of proMMP-2 in vivo. J Biol Chem.; 275: 26411–26415.

complex of proMMP-2-TIMP-2–MT1-MMP is then presented to an adjacent free MT1-MMP for activation (interactions of MT1-MMP on the cell surface through hemopexin domains facilitates the activation process[57]).

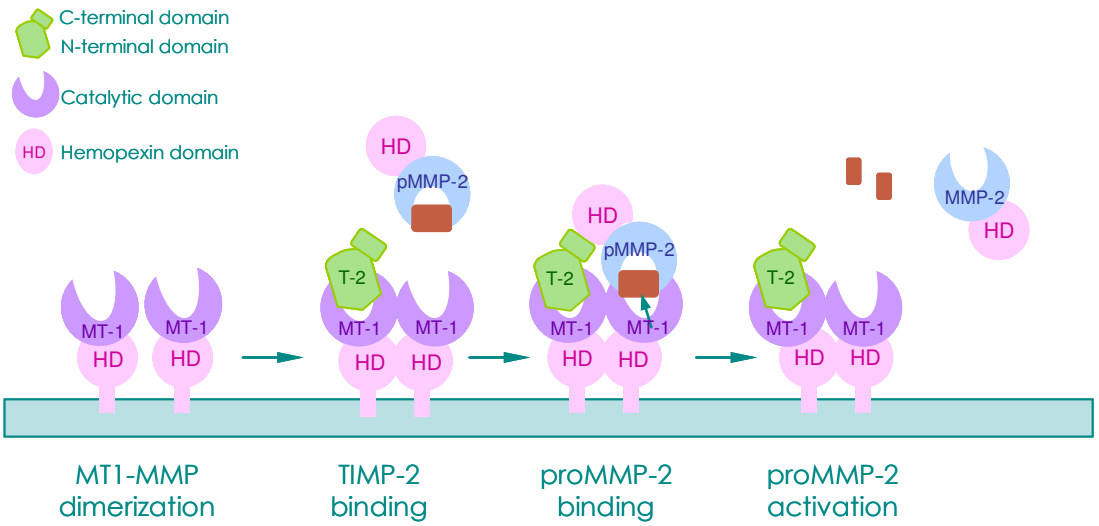


Figure 1.4 - Model of proMMP-2 activation by MT1-MMP and TIMP-2. Active MT1-MMP (MT-1) on the membrane binds a molecule of TIMP-2 (T-2), inhibiting its activity. MT1-MMP can form dimers or multimers on the cell surface through interaction of the hemopexin domains (HD). ProMMP-2 (pMMP-2) subsequently binds to the C-terminal domain of TIMP-2 through its hemopexin domain. The second, active, MT1-MMP then cleaves the bait region of proMMP-2, thereby partly activating it. The MMP-2 dissociates from the membrane and is fully activated by intermolecular processing (adapted from Visse & Nagase, 2003[36]).

Considerable progress has been made in the understanding of biochemical and structural aspects of MMPs, including their activation and catalytic mechanisms, substrate specificity, and the mechanism of inhibition by TIMPs. Nevertheless, there are important questions that remain unanswered. In general, MMPs play critical roles in many normal growth and developmental aspects of tissue remodeling, wound healing, and angiogenesis, and in many pathological situations, are associated with cell migration, invasion, arthritis, and cancer tumour progression. MMPs carry out a myriad of biological functions by acting simply on ECM protein scaffold or by orchestrate cell behaviour handing out some biologically active molecules, such as growth factors.

[57] Itoh Y, Takamura A, Ito N, Maru Y, Sato H, Suenaga N, Aoki T, Seiki M (2001). Homophilic complex formation of MT1-MMP facilitates proMMP-2 activation on the cell surface and promotes tumor cell invasion. EMBO J.; 20: 4782–4793.

As it can be attested by Table 1.2 examination, MMP-1 is responsible for cell activities such as cell migration or platelet aggregation while MMP-2 is associated with epithelial cell migration and promotes neurite growth.

Table 1.2 - Biological Activities Generated by MMP-Mediated Cleavage

Biological Effect	**Responsible MMPs**
Keratinocyte migration and reepithelialization	MMP-1
Osteoclast activation	MMP-13
Neurite outgrowth	MMP-2
Adipocyte differentiation	MMP-7
Cell migration	MMP-1,- 2, -3
Cell migration	MT1-MMP
Mammary epithelial cell apoptosis	MMP-3
Mammary epithelial alveolar formation	MMP-3
Epithelial-mesenchymal conversion (mammary epithelial cells)	MMP-3
Mesenchymal cell differentiation with inflammatory phenotype	MMP-2
Platelet aggregation	MMP-1
Generation of angiostatin-like fragment	MMP-3 MMP-7 MMP-9 MMP-12
Generation of endostatin-like fragment	MMPs
Enhanced collagen affinity	MMP-2, -3, -7, -9, -13 (but not MMP-1)
Kidney tubulogenesis	MT1-MMP
Release of bFGF	MMP-3, -13
Increased bioavailability of IGF1 and cell proliferation	MMP-1, -2, -3 MMP-11
Activation of VEGF	MMPs
Epithelial cell migration	MMP-2, MT1-MMP
Apoptosis (amnion epithelial cells)	Collagenase
Proinflammatory	MMP-1, -3, -9
Tumor cell resistance	MMP-9
Antiinflammatory	MMP-1, -2, -9
Antiinflammatory	MMP-1, -2, -3, -13, -14
Increased bioavailability of TGF-ß	MMP-2, -3, -7
Disrupted cell aggregation and increased cell invasion	MMP-3, MMP-7
Reduced cell adhesion and spreading	MT1-MMP, MT2-MMP, MT3-MMP
Fas receptor–mediated apoptosis	MMP-7
Reduced IL-2 response	MMP-9

1.1.1. MMPs in the physiology of the brain

Matrix metalloproteinases and ADAMs (a disintegrin and metalloproteinase) are part of a larger family of structurally related zinc-dependent metalloproteinases called metzincins. As it has been mentioned above, structurally, MMPs are divided in three domains: an amino-terminal propeptide region, an amino-terminal catalytic domain, and a carboxy-terminal domain that is involved in substrate binding. ADAMs have a prodomain, a metalloprotease region, a disintegrin domain for adhesion, a cysteine-rich region, epidermal-growth-factor repeats, a transmembrane module and a cytoplasmic tail.

The activity of MMPs is tightly regulated in several ways: at the level of transcription, by post-translational modifications such as proteolysis, and through the action of endogenous tissue inhibitors of metalloproteinases. The regulation of ADAMs is less well understood, although there is some evidence that the same three levels of regulation might control ADAM activity.

MMPs and ADAMs have been implicated in neuroinflammation and multiple sclerosis, in the pathogenesis of malignant gliomas, and in other neurological conditions such as stroke, viral infections and Alzheimer's disease. In the case of ADAMs, their role in these pathological states has begun to be explored, but the available literature is still in its infancy.

Although the well documented detrimental roles of metalloproteinases some of their functions in the CNS might be beneficial. For example, some metalloproteinases are expressed in the CNS during development, pointing to a possible role in brain maturation (appendix, Table 7.1, for details).

Until now, gelatinases A and B (MMP-2 and MMP-9) have been the most frequently investigated MMPs in the brain. MMP-2 mRNA, protein, and enzymatic activity have been detected in various brain structures and reported to be preferably astroglial in origin[58,59,60,61,62,63,64]. In addition, Planas and

[58] Szklarczyk A, Lapinska J, Rylsk, M, McKay RD, Kaczmarek L (2002). Matrix metalloproteinase-9 undergoes expression and activation during dendritic remodeling in adult hippocampus. J. Neurosci. 22: 920–930.

[59] Rivera, S Ogier C, Jourquin J, Timsit S, Szklarczyk AW, Miller K, Gearing AJ, Kaczmarek L, Khrestchatisky M. (2002). Gelatinase B and TIMP-1 are regulated in a cell- and time-dependent manner in association with neuronal death and glial reactivity after global forebrain ischemia. Eur. J. Neurosci. 15: 19–32.

[60] Phillips L, Reeves TM (2001). Interactive pathology following traumatic brain injury modifies hippocampal plasticity. Restor. Neurol. Neurosci. 19: 213–235.

[61] Wright JW, Masino AJ, Reichert JR, Turner GD, Meighan SE, Meighan PC, Harding JW (2003). Ethanol-induced impairment of spatial memory and brain matrix metalloproteinases. Brain Res. 963: 252–261.

co-workers[65] found MMP-2 also in some cortical neurons, and neuronal MMP-2 has also been described in the cerebellum, including the Purkinje neurons[62,63,66]. A potentially functional role of MMP-2 in post-lesion axonal sprouting and neuronal plasticity has been suggested by Reeves and colleagues, who reported that differentiation and functional plastic recovery of the entorhinal cortex projections to the hippocampal dentate gyrus are associated with increased MMP-2 activity, and that MMP inhibitor FN-439 prevents recovery[67,68].

In contrast to MMP-2, MMP-9 mRNA and protein expression[59,69,70] as well as its activity[59,62,63,69,71] have been detected in the hippocampus, cerebellum and cortex, predominantly in neurons. MMP-9 (at the protein and enzymatic activity levels) has been found mainly in cell bodies and dendrites[58,65]. Furthermore, *in vitro* studies showed MMP-9 protein expression in the neuronal processes of cultured hippocampal neurons[58]. A more limited level of MMP-9 expression has also been observed in glia, in particular in astrocytes and microglia, both *in vivo* and *in vitro*[72]. Szklarczyk *and co-workers*[58] and Zhang *and collegues*[63] have shown that kainic acid (KA) treatment results in upregulation of MMP-9 mRNA, protein and enzymatic activity in hippocampal dentate gyrus and neocortex early after seizures (6–24 h). The exclusive hippocampal localization of MMP-9 upregulation within the dentate gyrus suggests MMP-9 involvement in synaptic plasticity. Most interestingly, the enhanced mRNA expression was observed

[62] Zhang JW, Deb S, Gottschall PE (2000). Regional and age-related expression of gelatinases in the brains of young and old rats after treatment with kainic acid. Neurosci. Lett. 295: 9–12.
[63] Zhang JW, Deb S, Gottschall PE (1998). Regional and differential expression of gelatinases in rat brain after systemic kainic acid or bicuculline administration. Eur. J. Neurosci. 10: 3358–3368.
[64] Jourquin J, Tremblay E, Decanis N, Charton G, Hanessian S, Chollet AM, Le Diguardher T, Khrestchatisky M, Rivera S (2003). Neuronal activity-dependent increase of net matrix metalloproteinase activity is associated with MMP-9 neurotoxicity after kainate. Eur. J. Neurosci. 18: 1507–1517.
[65] Planas AM, Sole S, Justicia C (2001). Expression and activation of matrix metalloproteinase-2 and -9 in rat brain after transient focal cerebral ischemia. Neurobiol. Dis. 8: 834–846.
[66] Wright JW, Masino AJ, Reichert JR, Turner GD, Meighan SE, Meighan PC, Harding JW. Ethanol-induced impairment of spatial memory and brain matrix metalloproteinases. Brain Res. 2003 Feb 14;963(1-2):252-61.
[67] Phillips LL, Reeves TM (2001). Interactive pathology following traumatic brain injury modifies hippocampal plasticity. Restor. Neurol. Neurosci.19: 213–235.
[68] Reeves TM, Prins ML, Zhu J, Povlishock JT, Phillips LL (2003). Matrix metalloproteinase inhibition alters functional and structural correlates of deafferentation-induced sprouting in the dentate gyrus. J. Neurosci. 23: 10182–10189.
[69] Szklarczyk A, Lapinska J, Rylski M, McKay RD, Kaczmarek L (2002). Matrix metalloproteinase-9 undergoes expression and activation during dendritic remodeling in adult hippocampus. J. Neurosci. 22, 920–930.
[70] Taishi P, Sanchez C, Wang Y, Fang J, Harding JW, Krueger JM (2001). Conditions that affect sleep alter the expression of molecules associated with synaptic plasticity. Am. J. Physiol. Regul. Integr. Comp. Physiol. 28: R839–R845.
[71] Vaillant C, Meissirel C, Mutin M, Belin MF, Lund LR, Thomasset N. (2003). MMP-9 deficiency affects axonal outgrowth, migration, and apoptosis in the developing cerebellum. Mol Cell Neurosci. 24(2):395-408.

both in the neuronal cell bodies as well as in the dendritic layer, implying an activity-driven translocation of the MMP-9 mRNA[72].

Furthermore, MMP-9 mRNA expression levels were increased after KCl-depolarization in the cortex[72,73]. Enzymatic activity of MMP-9 was found to be elevated in the rat hippocampus and prefrontal cortex in the course of spatial learning[66], differing from MMP-2 activity. On the other hand, MMP-9 mRNA level decreases in the cerebral cortex after sleep deprivation whereas after enhanced ambient temperature, a condition that promotes sleep, it increases[70]. During postnatal cerebellar development, MMP-9 is expressed by granule and Purkinje neurons of the cerebellum. MMP-9 promotes cerebellar granule cell migration and axonal growth during early postnatal life[71].

In vivo studies of MMP-9-deficient mice revealed abnormal accumulation of granular precursors in the external granular layer at a time when migration is normally extensive. Furthermore, granular precursor migration was delayed and their programmed cell death was reduced in MMP-9-deficient mice, suggesting that MMP-9 is involved in the control of granule cell migration and apoptosis. These results provide direct evidence for a physiological role of MMP-9 in neuronal precursor migration and apoptosis in the developing cerebellum, and emphasize the importance of MMP-9 in the temporal regulation of the cerebellar microenvironment. These results suggest a potencial connection(s) between neuronal survival, synaptogenesis and MMP-9 since it has been shown a close association of MMP-9 expression with neuronal physiology. Apparently, no functional data have been reported specifically on MMP-9 and neuronal plasticity, despite the availability of MMP-9 knockout mice. Those animals were, however, used for brain damage studies, and it has been found that lack of MMP-9 reduces the neurodegeneration evoked by either ischemia or brain trauma[35,74,75,76]. This notion appears surprising in the context of the previously described data implicating MMP-9 in neuronal plasticity. Recently, Wang & Tsirka provided evidence that MMP-9 may play a deleterious role in acute brain

[72] Dzwonek J, Rylski M, Kaczmarek L. (2004). Matrix metalloproteinases and their endogenous inhibitors in neuronal physiology of the adult brain. FEBS Lett. 567(1):129-135. Review.

[73] von Gertten C, Holmin S, Mathiesen T, Nordqvist AC (2003). Increases in matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1 mRNA after cerebral contusion and depolarisation. J Neurosci Res. 73(6): 803-810.

[74] Asahina M, Yoshiyama Y, Hattori T (2001). Expression of matrix metalloproteinase-9 and urinary-type plasminogen activator in Alzheimer's disease brain. Clin Neuropathol. 20(2): 60-63.

[75] Yong VW, Power C, Forsyth P, Edwards DR (2001). Metalloproteinases in biology and pathology of the nervous system. Nat Rev Neurosci. 2(7):502-11. Review.

injury within the first 3 days after intracerebral haemorrhage and that blockade of MMP activity during this critical period may have efficacy as a therapeutic strategy for the treatment of acute brain injury[77]. However, although under the variety of neurodegenerative conditions (ischemia, brain trauma, viral infections, amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease) MMP-9 still remains mainly neuronal in origin, it is not clear whether it is expressed by the neurons prone to death and/or by those attempting to recover from the damage[37,59,74,75,76,77,78,79,80]. Other MMPs, such as MMP-25 (MT5-MMP) and MMP-3 (stromelysin-1) have been described by several researchers. Nevertheless their specific role in brain physiology remains to be elucidated.

Scheme A of Fig. 1.5 represents MMP-9 activation[72], showing several regulation steps. There are a number of gene regulatory sequences driving the MMP-9 expression. The transcription factors activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) are the most prominent. Once produced, the MMP-9 mRNA is subjected to regulation at its stability level by nitric oxide (NO); as well it can apparently be translocated in neurons towards activated dendrites. Notably, MMP-9 contains ARE-like sequences that have been suggested to be of pivotal importance for the translocation in other messages. The protein is synthesized with the signal peptide responsible for a translocation to the endoplasmic reticulum, where the signal peptide is removed. Glycosylation of MMP-9 protein provides an additional level of regulation. The next step is an export of MMP-9 protein, in vesicles, to the extracellular space. The MMPs are produced by cells in an inactive form (pro-MMP) where the cystein residue of the propeptide region is bond to a zinc atom present in the catalytic domain.

Fully active protein is produced by disruption of the cysteine–zinc interaction and enzymatic removal of the propeptide. The activation of the pro-enzyme is controlled by a cascade of steps involving other MMPs and the plasmin system.

---

[76] Cunningham LA, Wetzel M, Rosenberg GA (2005). Multiple roles for MMPs and TIMPs in cerebral ischemia. Glia. 50(4): 329-339. Review.

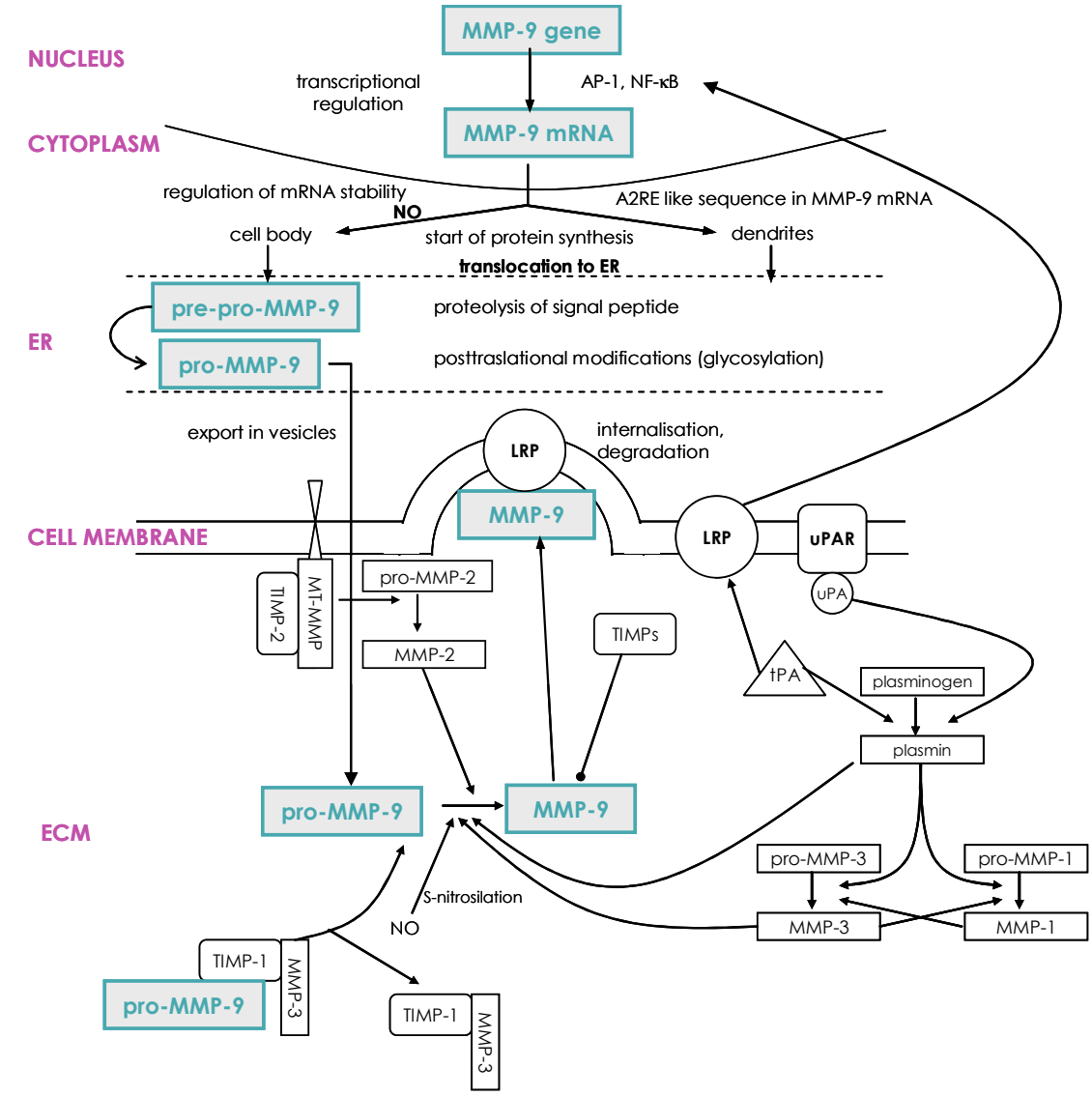
[77] Wang J, Tsirka SE (2005). Neuroprotection by inhibition of matrix metalloproteinases in a mouse model of intracerebral haemorrhage. Brain 128, 1622–1633.

[78] Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, Smith JW, Liddington RC, Lipton SA (2002). S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. Science. 297(5584):1186-1190.

[79] Lorenzl S, Albers DS, Narr S, Chirichigno J, Beal MF (2002). Expression of MMP-2, MMP-9, and MMP-1 and their endogenous counterregulators TIMP-1 and TIMP-2 in postmortem brain tissue of Parkinson's disease. Exp Neurol. 178(1):13-20.

[80] Khuth ST, Akaoka H, Pagenstecher A, Verlaeten O, Belin MF, Giraudon P, Bernard A. (2001). Morbillivirus infection of the mouse central nervous system induces region-specific upregulation of MMPs and TIMPs correlated to inflammatory cytokine expression. J Virol. 75(17): 8268-8282.

A



B

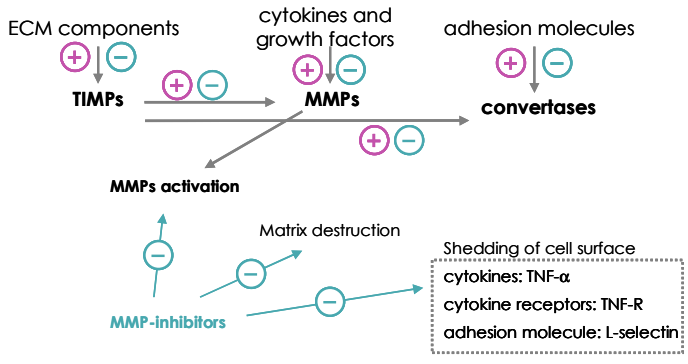


Figure 1.5 – (A) Schematic representation of MMP-9 activation (A2RE, A2 response element; AP-1, activator protein-1; ER, endoplasmic reticulum; LRP, low-density lipoprotein receptor-related protein; NF-κB, nuclear factor-κB; NO, nitric oxide; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; uPAR, uPA receptor **(B)** The network of MMPs/TIMPs, sheddases and their modulatory molecules as target of MMP inhibitors. (adapted from Dzwonek J *et al.*, 2004[72]).

Tissue type plasminogen activator (tPA), as well as urokinase type plasminogen activator (uPA) are principal enzymes releasing active plasmin that activates MMP-1 and MMP-3, which in turn can cleave pro-MMP-9. Interestingly, tPA can act on MMP-9 also in a plasmin-independent manner, namely it has been shown that tPA can bind to the low-density receptor related protein (LRP) and indirectly regulate MMP-9 gene transcription. LRP can also act as a receptor for MMP-9 that mediates internalization and degradation of the enzyme. Finally, MMP-9 activity can be inhibited by TIMPs.

Scheme B (Fig. 1.5) represents the network of MMPs/TIMPs and their modulatory molecules as target of MMPs inhibitors. TIMPs and MMPs are regulated by ECM components, cytokines and growth factors. The effect of this regulation may display a cascade of proteolytic events that ends with the degradation of the ECM. TIMPs have special role by blocking MMPs activity and, consequently, inhibiting matrix destruction.

This example illustrates the complexity and the widespread of the potential events that may occur during physiological and pathological cell development.

1.1.2. TIMPs in the physiology of the brain

All four TIMP genes have been shown to be expressed in unstimulated brain[81,82,83] (appendix, Table 7.2, for details). TIMP-1 mRNA and protein have been found to be present in the hippocampus[83]. TIMP-1 mRNA expression was also described in cortical neurons[73] and in the cerebellum[84], where Villant and co-workers[85] have found TIMP-1 protein in the cell bodies of interneurons,

[81] Vaillant C, Didier-Bazes M, Hutter A, Belin MF, Thomasset N (1999). Spatiotemporal expression patterns of metalloproteinases and their inhibitors in the postnatal developing rat cerebellum. J. Neurosci. 19: 4994–5004.

[82] Rathke-Hartlieb S, Budde P, Ewert S, Schlomann U, Staege MS, Jockusch H, Bartsch JW, Frey J (2000). Elevated expression of membrane type 1 metalloproteinase (MT1-MMP) in reactive astrocytes following neurodegeneration in mouse central nervous system. FEBS Lett. 481(3): 227-34.

[83] Rivera S, Tremblay E, Timsit S, Canals O, Ben-Art Y, Khrestchatisky M (1997). Tissue inhibitor of metalloproteinases-1 (TIMP-1) is differentially induced in neurons and astrocytes after seizures: evidence for developmental, immediate early gene, and lesion response. J. Neurosci. 17: 4223–4235.

[84] Fager N, Jaworski DM (2000). Differential spatial distribution and temporal regulation of tissue inhibitor of metalloproteinase mRNA expression during rat central nervous system development. Mech Dev. 98(1-2): 105-109.

[85] Vaillant C, Didier-Bazes M, Hutter A, Belin MF, Thomasset N (1999).Spatiotemporal expression patterns of metalloproteinases and their inhibitors in the postnatal developing rat cerebellum. J Neurosci. 19(12): 4994-5004.

Purkinje cells and Bergman glia. Nedivi and colleagues[86] were the first to find increased levels of TIMP-1 mRNA in the hippocampal dentate gyrus following seizures. Rivera and co-workers[83] and Jaworski and collegues[87] extended this observation, by documenting elevated TIMP-1 mRNA and protein expression in all of the hippocampal subfields, thus suggesting that it is a neuronal response to the enhanced activity. Furthermore, Rivera and co-workers[83] found that seizures elevate TIMP-1 immunoreactivity in neuronal cell bodies and dendritic areas at early stages after seizures and in astrocytes 3 days after seizures, when the reactive gliosis is present (as result of neuronal death and consequent formation of a dense fibrous network of neuroglia in areas of damage). Gertten and colleagues[73] have shown that expression of TIMP-1 mRNA increases in cortical neurons after KCl-depolarization. Robust induction of TIMP-1 mRNA has been observed after acute electroconvulsive seizures in neurons of the dentate gyrus of the hippocampus and the outer layer of the cerebral cortex[72]. In pathological conditions of brain damage evoked by ischemia as well as viral infections, elevation of TIMP-1 expression has also been noted and suggested to have a neuroprotective potential[72,76,77]. It is also of great interest that TIMP-1 gene expression in the hippocampus is regulated by AP-1 transcription factor, whose association with neuronal plasticity, including learning and memory, has been very extensively documented[72,87]. Even though, the possible function of TIMP-1 in the brain is concerned, very little is known. However, it should be noted that this protein delivered in an adenoviral vector was capable of inhibiting excitotoxic cell death in cultured neurons[88]. The action of TIMP-1 was anti-necrotic rather than anti-apoptotic as it was very fast (already within 1h) and specific to glutamate evoked neuronal loss, but not effective against chemical induced ischemia or nerve growth factor withdrawal. Furthermore, blocking MMP activity using synthetic MMP inhibitors as well as treatment with TIMP-3 offered no neuroprotection against excitotoxicity[88]. The possible mechanism of TIMP-1 neuroprotection involves its ability to lower intracellular calcium levels in response to glutamate. TIMP-2 is the most abundantly

[86] Nedivi E, Hevroni D, Naot D, Israeli D, Citri Y (1993). Numerous candidate plasticity-related genes revealed by differential cDNA cloning. Nature. 363(6431): 718-22.
[87] Jaworski J, Biedermann IW, Lapinska J, Szklarczyk A, Figiel I, Konopka D, Nowicka D, Filipkowski RK, Hetman M, Kowalczyk A, Kaczmarek L (1999). Neuronal excitation-driven and AP-1-dependent activation of tissue inhibitor of metalloproteinases-1 gene expression in rodent hippocampus. J Biol Chem. 274(40): 28106-28112.
[88] Tan HK, Heywood D, Ralph GS, Bienemann A, Baker AH, Uney JB (2003). Tissue inhibitor of metalloproteinase 1 inhibits excitotoxic cell death in neurons. Mol Cell Neurosci. 22(1): 98-106.

expressed TIMP in the adult CNS[84]. In the rat cerebellum both the mRNA and protein are present in cell bodies of interneurons, Purkinje cells and granular neurons in the internal granular layer[85]. In the cortex, expression of TIMP-2, mRNA is observed predominantly in neurons[72,73]. Notably, TIMP-2 expression appears to be upregulated neither by neuronal depolarization nor by pathological conditions that activate TIMP-1[79]. TIMP-3 is expressed at the lowest level in the adult rat brain[84], although its mRNA has been found in the cortex[89], cerebellum[85], thalamus, olfactory bulb[84], and brain stem (composite substructure of the brain that includes the midbrain, the pons and the medulla oblongata)[83].

Immunohistochemistry has shown cerebellar TIMP-3 expression in the dendritic elongations of Purkinje cells and in the granular neurons in the internal granular layer. In contrast, TIMP-3 mRNA was only detected in the cell bodies of Purkinje cells. During viral infections there is TIMP-3 mRNA and protein upregulation in neurons within hours[90]. TIMP-3 protein is produced by primary cultures of cortical neurons[91] and astrocytes[92]. Functionally, it has been shown that TIMP-3 may play a proapoptotic role in a model of neuronal cell death evoked by doxorubicin[91]. The least is known about TIMP-4 expression in the brain. TIMP-4 mRNA is present in the cerebellum, cerebrum (telencephalon) and brain stem[84].

MMPs are effectors of blood brain barrier opening and invasion of brain parenchyma by immune cells in multiple sclerosis and brain metastasis. In addition they can act, together with related metalloproteinases, as enhancers of the immune response via their proteolytic release of membrane-bound cytokines and their receptors. Hydroxamic acid type MMP-inhibitors have been used successfully in animal models of multiple sclerosis and brain metastasis to attenuate acute disease symptoms and structural damage of the brain

[89] Gardner J, Ghorpade A (2003).Tissue inhibitor of metalloproteinase (TIMP)-1: the TIMPed balance of matrix metalloproteinases in the central nervous system. J Neurosci Res. 74(6):801-6. Review.

[90] Khuth ST, Akaoka H, Pagenstecher A, Verlaeten O, Belin MF, Giraudon P, Bernard A (2001).Morbillivirus infection of the mouse central nervous system induces region-specific upregulation of MMPs and TIMPs correlated to inflammatory cytokine expression. J Virol. 75(17):8268-82.

[91] Wetzel M, Rosenberg GA, Cunningham LA (2003). Tissue inhibitor of metalloproteinases-3 and matrix metalloproteinase-3 regulate neuronal sensitivity to doxorubicin-induced apoptosis. Eur J Neurosci. 18(5): 1050-1060.

[92] Muir EM, Adcock KH, Morgenstern DA, Clayton R, von Stillfried N, Rhodes K, Ellis C, Fawcett JW, Rogers JH (2002). Matrix metalloproteases and their inhibitors are produced by overlapping populations of activated astrocytes. Brain Res Mol Brain Res. 100(1-2):103-117.

parenchyma[93,94]; in brain metastasis they were able to attenuate postmeningitidal cognitive deficits. A large number of potent inhibitors have been discovered and some have been tested in clinical trials as therapies for cancer and arthritis with great expectations[95]. Although many of these experiments were discontinued, a few MMP inhibitors are still undergoing clinical trials. Most preclinical studies have focused on the role of MMPs in the early stages (progression and metastases) of cancer, in which MMP inhibition seems to have its greatest effect. Unfortunately, clinical trials of MMP inhibitors were conducted almost uniformly in patients with advanced, metastatic disease, and all have failed to show any beneficial effect on patients. It is possible that these same trials could have had different outcomes if they were conducted in patients with earlier stages of disease. The use of this novel group of compounds in human diseases requires a better understanding of the array of MMPs involved in the pathogenesis of a specific disease and the design of inhibitors with the respective specific target profile. Although not treating a primary cause of disease, their disease-modifying properties make them primary candidates for adjuvant and symptomatic therapy in multiple sclerosis and brain metastasis, respectively[96].

1.1.3 MMPs target molecules in the brain ECM

The ECM is composed of secreted glycoproteins and proteoglycans that form a network to which cells adhere. In the CNS this network accounts for upwards 20% of the total volume and consists predominantly of the proteins fibronectin, laminin, vitronectin, thrombospondin, tenascin, and collagen IV[97].

The ECM transmits signals from cells to the ECM and vice versa and integrins have a central role in these signalling pathways[98]. Over the past few years, evidence has emerged that integrins are crucial to brain development,

[93] Skotnicki JS, Zask A, Nelson FC, Albright JD, Levin JI (1999). Design and synthetic considerations of matrix metalloproteinase inhibitors. Ann N Y Acad Sci. 878:61-72. Review.
[94] Nelson AR, Fingleton B, Rothenberg ML, Matrisian LM (2000). Matrix Metalloproteinases: Biologic Activity and Clinical Implications. J Clin Oncol. 18(5):1135-64.
[95] Govinda Rao B (2005). Recent developments in the design of specific matrix metalloproteinase inhibitors aided by structural and computational studies. Current Pharmaceutical Design, 2005, 11, 295-322.
[96] Leppert D, Lindberg R, Kappos L and Leib S. (2001) Matrix metalloproteinases: multifunctional effectors of inflammation in multiple sclerosis and bacterial meningitis. Brain Research Reviews, 36 (2-3): 249-257
[97] Wright JW, Harding JW. (2004). The brain angiotensin system and extracellular matrix molecules in neural plasticity, learning, and memory. Prog Neurobiol. 72(4):263-93. Review.
[98] Van der Flier A, Sonnenberg A. Function and interactions of integrins. Cell Tissue Res 2001; 305 (3): 285-298.

performing more than just adhesion molecules, they exert control over various signalling pathways to regulate virtually every aspect of cell behaviour.

This so-called "perineuronal net" appears to provide physical support, regulate ionic and nutritional homeostasis of surrounding cells, and possesses ligands that interact with cell surface receptors that initiate signalling events designed to guide a wide range of functions including cellular proliferation, motility, differentiation, neurite outgrowth, growth cone targeting, synapse stabilization, and apoptosis[97,99,100].

MMPs seam to process their substrates following a peculiar manner; it is not clear what restrictions may modulate substrate recognition and activity. There are virtually no well documented physiological MMP target proteins, but several of pathological ones. Recently, in the context of ECM the expression "cryptic" (from the Latin word *crypticus* and the Greek word *kryptikos*) refers to functional sites that are buried inside the structure of the ECM macromolecules. Activation of these cryptic ECM activities requires structural modification, which can occur by conformational change or proteolytic processing. The chief enzymes that cleave matrix components, exposing these cryptic sites, are the MMPs.

Yet the brain is largely devoid of classical basement membranes – it does, however, have extracellular matrix molecules[101].

Thus, MMPs are important components in many biological and pathological processes because of their ability to degrade ECM components. It has become clear that ECM is not a mere scaffold for cells but that it also harbours cryptic biological functions that can be revealed on proteolysis. This puts a new light on the interplay between cells, the ECM, and its catabolism. In the neurons of the brain several components of the TIMP/MMP system are expressed and are responsive to changes in neuronal activity. Furthermore, functional studies, especially involving blocking of MMP activities, along with the identification of MMP substrates in the brain strongly suggest that this enzymatic system plays an important physiological role in adult brain neurons, possibly being pivotal for neuronal plasticity.

---

[99] Webber CA, Hocking JC, Yong VW, Stange CL, McFarlane S (2002). Metalloproteases and guidance of retinal axons in the developing visual system. J. Neurosci. 22: 8091–8100.

[100] Shubayev VI, Myers RR (2004). Matrix metalloproteinase-9 promotes nerve growth factor-induced neurite elongation but not new sprout formation in vitro. J. Neurosci. Res. 77: 229–239.

[101] Novak, U. and A.H. Kaye (2000). Extracellular matrix and the brain: components and function. Review. J. Clin. Neurosci. 7, 280-290.

1.2 Cell culture

1.2.1 Types of cell cultures

The most truthfully and viable way to assess signalling pathways and regulation mechanisms of cell biology is throughout skills involved in cell culture. Cell culture has been widely used as an essential tool in molecular biology, toxicology and biotechnology, to address important technical and economical aspects of biology. There are three varieties of cultured cells - primary cultures, cell strains and cell lines - that will be succinctly described in the next paragraphs. It will be emphasised the advantageous aspects of each type of culture.

Primary culture is derived either by outgrowth of migrating cells from a fragment of tissue or by enzymatic or mechanical dispersal of the tissue. Regardless of the method employed, this is the first in a series of selective processes that have might untimely give rise to a relatively uniform cell line. In primary explantation selection occurs by virtue of the cells capacity to migrate from the explant while with dispersed cells, only those cells that survive the disaggregation technique and adhere to the substrate or survive in suspension will form the basis of a primary culture. The preparation of primary cultures is labour intensive and they can be maintained *in vitro* only for a limited period of time. During their relatively limited life span primary cells usually retain many of the differentiated characteristics of the cell *in vivo*, which allows a more closely prediction of reality cell behaviour of physiological or pathological states[102].

If the primary culture is maintained for more than a few hours, a further selection step will occur. Cells capable of proliferation will increase, some cell types will survive but not increase, and yet others will be unable to survive under particular conditions used. Hence, the relative proportion of each cell type will change and continue to do so until, in the case of monolayer cultures, all the available culture substrate is occupied. Primary cultures are derived directly from the tissue of a living organism. The development of this technique in the early 20th century by Harrison[103,104,105,106] marked the first experiments using

[102] Peehl DM (2004). Are primary cultures realistic models of prostate cancer? J Cell Biochem., 91(1):185-95.
[103] Harrison RG. 1906. Further experiments on the development of peripheral nerves. Amer J Anat 5:121–131.
[104] Harrison RG. 1907. Experiments in transplanting limbs and their bearings on the problems of the development of nerves. J Exp Zool 4:239–281.

cultured cells. In fact, in 1910 in a single stroke Harrison invented the method of tissue culture, and then used it to prove the neuron doctrine[105]. In these experiments, explants of tissue from spinal cord of a frog embryo were incubated in lymphatic fluid. After a day in culture, light microscopy was used to observe that the neurons sprouted axons.

Cell strains are derived from primary cultures by dissociating the cells from one another and plating them onto plastic tissue culture dishes. This type of culture, also called continuous culture, is comprised of a single cell type that adheres to the dishes and grows and divides when incubated in media. Many cell strains retain the differentiated properties characteristic to their tissue of origin. Cell strains can be serially propagated in culture either for a limited number of cell divisions (approximately thirty) or otherwise indefinitely.

Cell lines consist in a population of cultured cells that have undergone genetic changes that allow them to divide indefinitely – i.e. cells have become "immortal". Continuous cell lines that can be propagated indefinitely generally have this ability because they have been transformed into tumour cells. Tumour cell lines are often derived from actual clinical tumours, but transformation may also be induced using viral oncogenes or by chemical treatments. Transformed cell lines present the advantage of almost limitless availability, but the disadvantage of having retained very little of the original *in vivo* characteristics.

1.2.2 Cell culture scene – the therapeutic and technological challenges in neuroscience

Considering 1.5 kilograms of flaccid matter, elaborated folds, about 100 billion neuronal components, hundreds of trillions of interconnections, many thousand kilometres of cabling… The brain is often described as the most complex system in the universe. The task for neuroscientists is to assess how complexity can help scientific community to better understand the mechanism of nervous systems[107].

[105] Harrison RG. 1910. The outgrowth of the nerve fiber as a mode of protoplasmic movement. J Exp Zool 9:787–846.
[106] Keshishia H (2004). Ross Harrison's ''The Outgrowth of the Nerve Fiber as a Mode of Protoplasmic Movement'' J Exp Zool 301A:201–203.
[107] Koch C, Laurent G (1999). Complexity and the Nervous System. Science, 284(5411): 96 – 98.

Cultured neurons artificially prevented from expressing a natural dynamic behaviour can rapidly modify their molecular makeup and revert to their original activity pattern[108]. Synaptic properties also are bafflingly varied. Chemical synapses show a host of plastic phenomena whose time-courses span at least nine orders of magnitude, from milliseconds to weeks, providing a substrate for learning and memory[107].

In summary, no brain, however small, is structurally simple. The establishment of primary cell cultures is invaluable for studying cell and molecular biological questions. A variety of human cell lines exist, however, as described above, most have acquired significant genetic alterations from their cells of origin, including deletion of important regulatory genes. Therefore is important to create new methods to ascertain natural cell behaviour.

Presently, cell culture investigation, on the neuroscience field, have as primary propose to elucidate cell death mechanisms involved in acute cellular injury, necrosis, and programmed cell death to improve neuronal transplantation methodology, as well as in the development of therapeutic strategies for neuroprotection/neurorepair for patients with neurodegenerative diseases[109].

Although exciting, there are certain drawbacks to therapeutic possibilities based on organ/tissue transplantation. One of the most serious might be the immune response to foreign tissue, even when anti-rejection drugs are available, as they can have severe side-effects in certain patients. In 1998 the world woke up to the discovery that human embryonic stem (hES) cells could be grown in culture. A research group from Geron Company, published in Science[110] that they had successfully cultured hES cells and that these cells could originate all the major tissue types of the body. These new findings allow the removal of adult stem cells from patient under treatment, expand these in the culture plate, genetically modify them, and finally transplant them back. Another important discover in neurological field, that opened the perspectives for overlap many degenerative diseases, was made by Kempermann and Gage[111]. In 1999, they found dividing cells in the human adult brain, that can

[108] Turrigiano G, LeMasson G, Marder E (1995) Selective regulation of current densities underlies spontaneous changes in the activity of cultured neurons. J Neurosci 15:3640-3652.
[109] Boonman Z, Isacson O (1999). Apoptosis in Neuronal Development and Transplantation: Role of Caspases and Trophic Factors Experimental Neurology,156(1): 1-15.
[110] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science. 1998 Nov 6;282(5391):1145-7
[111] Kempermann G, Gage FH (1999) Experience-dependent regulation of adult hippocampal neurogenesis: effects of long-term stimulation and stimulus withdrawal. Hippocampus 9:321–332.

grow in the culture dish. The idea of using cells rather than whole organs to alleviate disease has led to the term "cell therapy". Presently, the major problem persists: the origin of the cell. By combining genetics with cell biology scientific community controls life process, getting closer to playing God. This scenario will throw up some of the most important philosophical, moral, ethical and theological questions. Clearly, cell biology could deliver therapy for many illness people, and may contribute to future discoveries regarding human health, but in the process of generating human embryos or continually replace body tissues will open a Pandora's box of ethical and moral issues.

Together bioethical concepts and technological challenges have provided alternative routes on cell-based therapies for human brain disease.

Feron and his research team (2005[112]) demonstrated the feasibility of taking a nasal biopsy and reliably generating enough cells in culture for transplantation within 4 weeks as therapeutic strategy. They concluded that transplantation of autologous olfactory ensheathing cells into the injured spinal cord is feasible and is safe up to one year post-implantation.

Similarly, Westerlund and co-workers (2005[113]) stated that it may be feasible to produce neural tissue for autotransplantation from endoscopically harvested stem cells, but further work is needed in refining culture protocols to control phenotype fate. These recent observations sustain the development of new protocols of cellular isolation and culturing that, consequently improve cell yield, viability and recover.

1.2.3. Enzymes used for brain disaggregation

1.2.3.1 Trypsin

A. General considerations

Trypsin is a prominent member of the serine protease family, and forms the functional principle of some large and highly specific proteases, involved in processes ranging from digestion to key regulatory mechanisms such as

[112] Feron F, Perry C, Cochrane J, Licina P, Nowitzke A, Urquhart S, Geraghty T, Mackay-Sim A. (2005). Autologous olfactory ensheathing cell transplantation in human spinal cord injury. Brain. 128(Pt 12):2951-60.

coagulation and hormone release, that serve to maintain a favourable extracellular environment[114].

Trypsin is an endopeptidase, i.e. cleavage occurs within the polypeptide chain rather than at the terminal amino acids located at the ends of polypeptides. This pancreatic serine protease specifically cleaves at the carboxylic side of the positively charged lysine and arginine residues[115]. Restrictions to the specificity of trypsin occur when proline is at the carboxylic side of lysine or arginine; the bond is almost completely resistant to cleavage by trypsin. Cleavage may also be considerably reduced when acidic residues are present on either side of a potentially susceptible bond[116]. Trypsin is derived from trypsinogen, the inactive precursor zymogen of trypsin, after enzymatic removal of an N-terminal 6-amino acid leader sequence resulting in the 23.8 kDa trypsin molecule. The optimum pH is 8.0 and it migrates as a single band on SDS-PAGE. Trypsin is inhibited by organophosphorus compounds such as diisopropylfluorophosphate and by natural inhibitors from pancreas. Soybean, lima bean, and egg white are also sources of natural inhibitors[117,118].

After its production in the pancreas, trypsin is transported to the small intestine, where begins the digestion of proteins to polypeptides and amino acids. Trypsin is an autocatalytic enzyme; that is, it catalyses the conversion of trypsinogen to trypsin although a small amount of another enzyme, enterokinase, is required to catalyze the initial reaction of trypsinogen to trypsin.

This pancreas-derived enzyme has been increasingly applied for protein sequencing, mapping and structure studies. For these kinds of applications, where the specificity of cleavage is exceptionally important, trypsin is often modified by TPCK, tosyl phenylalanyl chloromethyl Ketone, which will inactivate chymotrypsin (that is frequently present in trypsin solutions).

---

[113] Westerlund U, Svensson M, Moe M, Varghese M, Gustavsson B, Wallstedt L, Berg-Johnsen J, Langmoen I. (2005) Endoscopically Harvested Stem Cells: A Putative Method in Future Autotransplantation. Neurosurgery. 57(4):779-784

[114] Horn, H. and Heidland, A. (eds.) (1982) Proteases: Potential Role in Health and Disease, Plenum Press, New York and London

Cunningham, D.D. and Long, G.L. (eds.) (1987) Proteinases in Biologic Control and Biotechnology, Alan R. Liss, New York

Patthy, L. (1990). Evolutionary assembly of blood coagulation proteins. Semin. Thromb. Hemostasis 16, 245-259.

[115] Olsen JV, Ong S-E, Mann M (2004). Trypsin cleaves exclusively c-terminal to arginine and lysine residues. Molecular & Cellular Proteomics 3: 608–614, 2004.

[116] Wilkinson JM (1986). Fragmentation of Polypeptides by Enzymic Methods. In: Practical Protein Chemistry: A Handbook. A. Darbre, ed., John Wiley and Sons, New York, N.Y.

[117] Kostka V, Carpenter FH (1964). Inhibition of Chymotryptic Activity in Crystalline Trypsin Preparations. J. Biol. Chem., 239, 1799.

[118] Walsh KA (1970). Trypsinogens and Trypsins of Various Species in Methods in Enzymology, Vol. XIX, (Perlmann, G.E., and Lorand, L., eds.) pg, 41, Academic Press, New York.

B. Trypsin in cell culture

Trypsin is commonly used for the dissociation and disaggregation of anchorage-dependent mammalian cells and tissues, being extensively used in the laboratorial experiments to re-suspend cells adherent to the petri dish wall during the process of harvesting cells. The concentration of trypsin necessary to dislodge cells from their substrate is dependent primarily on the cell type and the age of the culture. Although treatment of cells with trypsin does not affect cellular viability to any great extent, trypsinization has been reported, since 1970s[119], to alter certain properties of the cell, including modification of adhesive properties, liberation of glycoproteins and sugars from the cell surface, among other effects that conditioned cell recover. The concentration of trypsin necessary to dislodge cells from their substrate is dependent primarily on the cell type and the age of the culture.

Autolysis constitutes the main inconvenient for trypsin usage in the laboratory. To minimize this problem users are encourage to store trypsin at cold temperatures (between −20 and −80°C). Another way of preventing trypsin self-cleavage is storage at pH 3. Activity is restored when the pH is adjusted back to pH 8.

Some aspects of trypsin activity are far to be overcome, for instance the low reproducibility of the commercial batches, witch can compromise the correct interpretation of experimental results.

1.2.3.2 Aspartic proteinases from *Cynara cardunculus* L.

Cardosins are aspartic proteinases (EC 3.4.23) isolated from the inflorescences of *Cynara cardunculus* L., whose milk-clotting activity has been exploited in Portugal in the manufacture of traditional cheeses since the Roman era.

Faro and co-workers[120] showed that the clotting activity of the aqueous extract of *C. cardunculus* pistils is due to the presence of an acid proteinase. Further studies revealed that the enzymatic extract is heterogeneous and that it

[119] Hodges GM, Linvingston DC, Franks LM (1973). The localization of trypsin in cultured mammalian cells. J. Cell Sci. 12: 887.

[120] Faro, C, Alface, J, Pires, E. 1987. *Cienc Biol.* 12 (5A): 201.

contains different aspartic proteinases, cardosin A_0[121], cardosin A and cardosin B[122,123].

Although, most of the biological functions proposed for cardosins are still speculative, cardosin A was shown to be highly expressed in the stigmatic papillae and to contain an Arg-Gly-Asp (RGD) motif which is a well known integrin-binding sequence allowing to suggest that this enzyme may participate in an RGD-dependent proteolytic mechanism in pollen–pistil interaction[124,125]. The specific localization of cardosin B in the stylar transmitting tissue also suggests that this enzyme may be involved in the remodelling and/or degradation of the pistil extracellular matrix during pollen tube growth[126]. Hence, it is possible that cardosins may fulfil important roles during the sexual reproduction of the plant.

Cardosin A_0, cardosin A and cardosin B are active at pH values between 2 and 7 with the maximum of activity determined to be close to pH 5. This characteristic, along with the fact that diazoacetil norleucine methyl ester (DAN) and pepstatin A inhibits both of them, clearly indicates that, as mentioned above they belong to the class of aspartic proteinases. Pepstatin inhibition constants are 3nM for cardosin A and less than 1nM for cardosin B[123]. Cardosins are two-chain glycosilated enzymes, which are though to have arisen by gene duplication[123].

They are synthesized as single-chain zymogens, and subsequently converted into two-chain mature enzymes[127,128]. The sequence alignment of cardosins A and B suggests that processing of the single-chain precursor, to produce the two-chain enzyme, is likely to occur through the cleavage of two peptide bonds, resulting in the removal of a region whose sequence is specific for plant

---

[121] Sarmento AS, Lopes H, Oliveira CS, Vitorino R, Amado F, Domingues P, Pires E, Domingues MRM, Barros MT. Purification and characterisation of new aspartic proteinases from *Cynara cardunculus* L. (*submitted article*).

[122] Faro C, Veríssimo P, Lin Y, Tan, J, Pires E (1995). Cardosin A and B, aspartic Proteases from the flowers of cardoon. Adv Exp Med Biol. 362:373-377.

[123] Veríssimo P, Faro C, Moir A, Lin Y, Tang J, Pires E (1996). Purification, characterisation and partial amino acid sequencing of two new aspartic proteinases from fresh flowers of Cynara cardunculus L. Eur J Biochem. 235: 762-768.

[124] Ramalho-Santos M, Pissarra J, Verissimo P, Pereira S, Salema R, Pires E, Faro CJ (1997) Cardosin A, an abundant aspartic proteinase, accumulates in protein storage vacuoles in the stigmatic papillae of *Cynara cardunculus* L. Planta 203, 204–212.

[125] Faro C, Ramalho-Santos M, Vieira M, Mendes A, Simões I, Andrade R, Verissimo P, Lin X, Tang J, Pires E (1999). Cloning and characterization of cDNA encoding cardosin A, an RGD-containing plant aspartic proteinase. J. Biol. Chem. 274, 28724–28729.

[126] Vieira M, Pissarra J, Verissimo P, Castanheira P, Costa Y, Pires E, Faro C (2001). Molecular cloning and characterization of cDNA encoding cardosin B, an aspartic proteinase accumulating extracellularly in the transmitting tissue of Cynara cardunculus L. Plant Mol. Biol. 45, 529–539.

[127] Simões I, Faro C (2004). Structure and function of plant aspartic proteinases. Eur.J. Biochem. 271: 2067-2075.

aspartic proteinases (PSI). This sequence, of about 100 amino acids, is present only in plant aspartic proteinases and bears no sequence similarity with aspartic proteinases of microbial or mammalian origin and its removal constitutes a crucial step in the regulation of cardosins activity.

Although they cleave preferably peptide bonds between residues with bulky hydrophobic side chains, cardosins B displays a broader specificity and a higher proteolytic activity[123,129130]. This has been demonstrated, for example, by Ramalho-Santos and colleagues in 1996. These studies pertaining to the independent action of cardosins A and B toward bovine caseins, made it clear that in α_{s1}-casein the two enzymes cleave, first, the bonds Phe_{23}-Phe_{24}, Phe_{153}-Phe_{154} and Trp_{164}-Tyr_{165}. Cardosin A also cleaves the bond Tyr_{165}-Tyr_{166}, whereas cardosin B cleaves an extra type of bond, Phe_{150}-Arg_{151}, revealing a slightly broader specificity[129]. Simões, 1998 developed similar work with β-casein and the differences on cardosins specificity were confirmed.

Several studies have been developed concerning their physicochemical characterisation. At the present the tree-dimensional structure of cardosin A is solved[131] and a large amount of information concerning its activity and specificity is available. Furthermore, cardosin A has been characterized regarding catalytic and specificity aspects in non conventional environments[132,133] and concerning its thermal stability using biophysical approaches[134].

A recent investigation has demonstrated that cardosin A cleaves collagen within the triple helix (Fig. 1.6), following a well defined pattern of hydrolysis that is maintained for at least 24 hours of digestion (as shown in Fig. 1.7-A),

[128] Ramalho-Santos M, Veríssimo P, Cortes L, Samyn B, Van Beeumen J, Pires E, Faro C (1998). Identification and proteolytic processing of procardosin A. Eur J Biochem. 255(1), 133-138.

[129] Ramalho-Santos M, Veríssimo P, Faro C, Pires E (1996). Action on bovine αs1 casein of cardosins A and B, aspartic proteinases from the flowers of the cardoon *Cynara cardunculus* L.. Biochim Biophys Acta 1297: 83-89.

[130] Simões I (1998). Caracterização molecular da acção das cardosinas A e B sobre caseínas α- e κ - bovinas. Dissertação de mestrado em Biologia Celular. Universidade de Coimbra. Coimbra, Portugal.

[131] Frazão C, Bento I, Costa J, Soares C, Veríssimo P, Faro C, Pires E, Cooper J, Carrondo M (1999). Crystal structure of cardosin A, a glycolsylated and Arg-Gly-Asp-containing aspartic proteinase from the flowers of Cynara cardunculus L. J Biol Chem. 274: 27694-27707.

[132] Sarmento AC, Oliveira CS, Pires EM, Halling PJ, Barros MT (2004). Evaluation of cardosin A as a proteolytic probe in the presence of organic solvents. J. Mol. Catal. B: Enzymatic, 31 (4-6): 137-141.

[133] Sarmento AC, Oliveira CS, Halling PJ, Pires E, Barros M (2003). Cardosin A as a model aspartic proteinase for the study of organic solvent effects. I. An overview on catalytic and structural aspects. J. Mol. Catal. B, Enzymatic 21, 19-23.

suggesting that cardosin A can hydrolyse collagen at a small number of specific peptide bonds. N-terminal sequencing of hydrolysis products allowed the identification of one cleavage site as being Phe_{464}-Gln_{465} in the α2 chain of collagen[135]. Cardosin A shows collagenolytic activity, directed within the helical region, hitherto believed to be resistant to all non-mammalian proteinases.

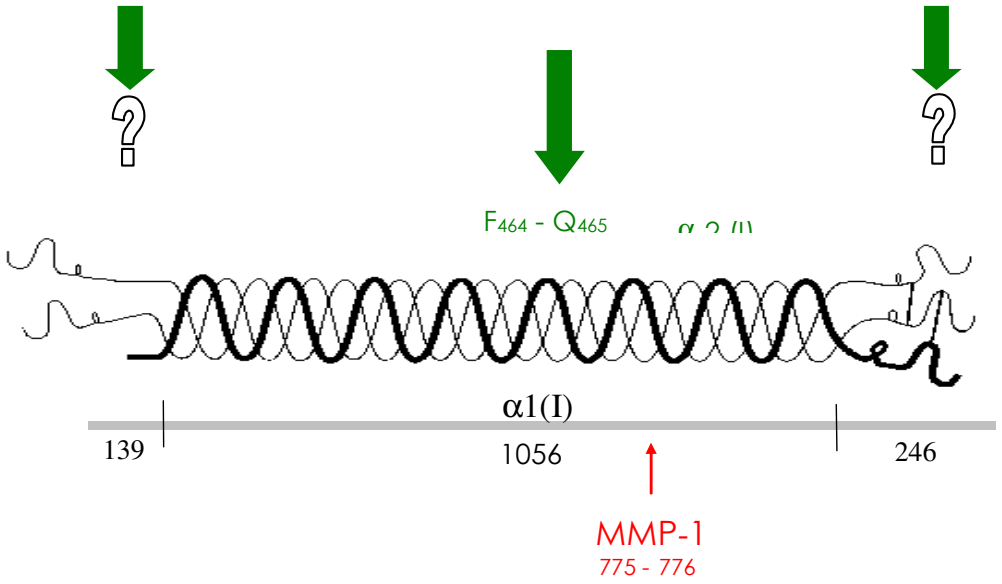


Figure 1.6 – Schematic representation of collagenolytic activity of cardoisn A (green arrows). The cleavage bond F_{464}-Q_{465} of alpha 2 of collagen type I was determined by sequence analysis. The traditional fragments TC^A and TC^B produced by collagenases are generated through cleavage at Gly_{775}-Leu/Ile_{776} bonds of triple helices (red arrow). (adapted from Duarte *et al.*, 2005[135])

It was additionally demonstrated, that cardosins A_0 and B can also hydrolyse human type I collagen[136]. The pattern of type I collagen hydrolysis by cardosins are shown in Fig. 1.7. It is possible to visualise the wide specificity of cardosin B towards collagen molecule, given that fragments produced at early incubation times are subsequently digested, in contrast to cardosins A_0 and A performance.

[134] Oliveira CS, Pina D, Sarmento AC, Pires E, Barros M, Villar H, Shnyrov V (2003). The structural stability of cardosin A of Cynara carduncullus L. Thermochimica Acta 71218: 1-12

[135] Duarte AS, Pereira A, Cabrita AS, Moir A, Pires E, Barros, M (2005). The characterisation of the collagenolytic activity of cardosin A demonstrates its potential application for extracellular matrix degradative processes. Current Drug Discovery Technologies. 2(1): 37-44.

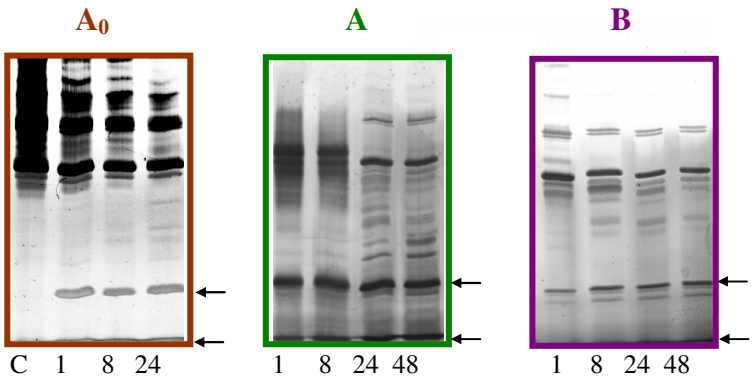


Figure 1.7 – Human type I collagen hydrolysis pattern generated by cardosins A_0, A and B (C: indigested collagen; 1, 8, 24 and 48 stand for incubation times in hours). Arrows represent the two subunits of each respective enzyme. (adapted from Duarte, 2001[136])

It was also shown, by using an animal model of intestinal adhesion produced by normal surgical procedures within the abdominal cavity, that immobilised cardosin A reduces the amount of fibrotic tissue that is formed after surgical intervention[137].

Concerning cardosin A_0 and cardosin B there is still little information but it is slowly being build up. At the present, a detailed characterisation of cardosin A_0 is being carried out. It is known that cardosin A_0 is in fact a heterogeneous mixture of enzymes that, on the contrary to what was thought until now, exhibit high proteolytic activity[121].

A simple purification protocol for these proteinases was developed and includes an acidic extraction followed by gel filtration and ion exchange chromatography[123]. Recently, the previously existent purification method was modified in order to achieve accurate and faster cardosins purification[132]. As represented in Fig. 1.8 cardosins mixture obtained by size-exclusion chromatography is further purified by ion-change chromatography to obtain cardosins A_0, A and B in an approximated proportion of 54, 41 and 5% of total protein in the fraction obtained by size-exclusion chromatography.

[136] Duarte AS (2001). Utilização de uma enzima vegetal na prevenção de fibroses e aderências. Dissertação de Mestrado. Universidade de Aveiro.

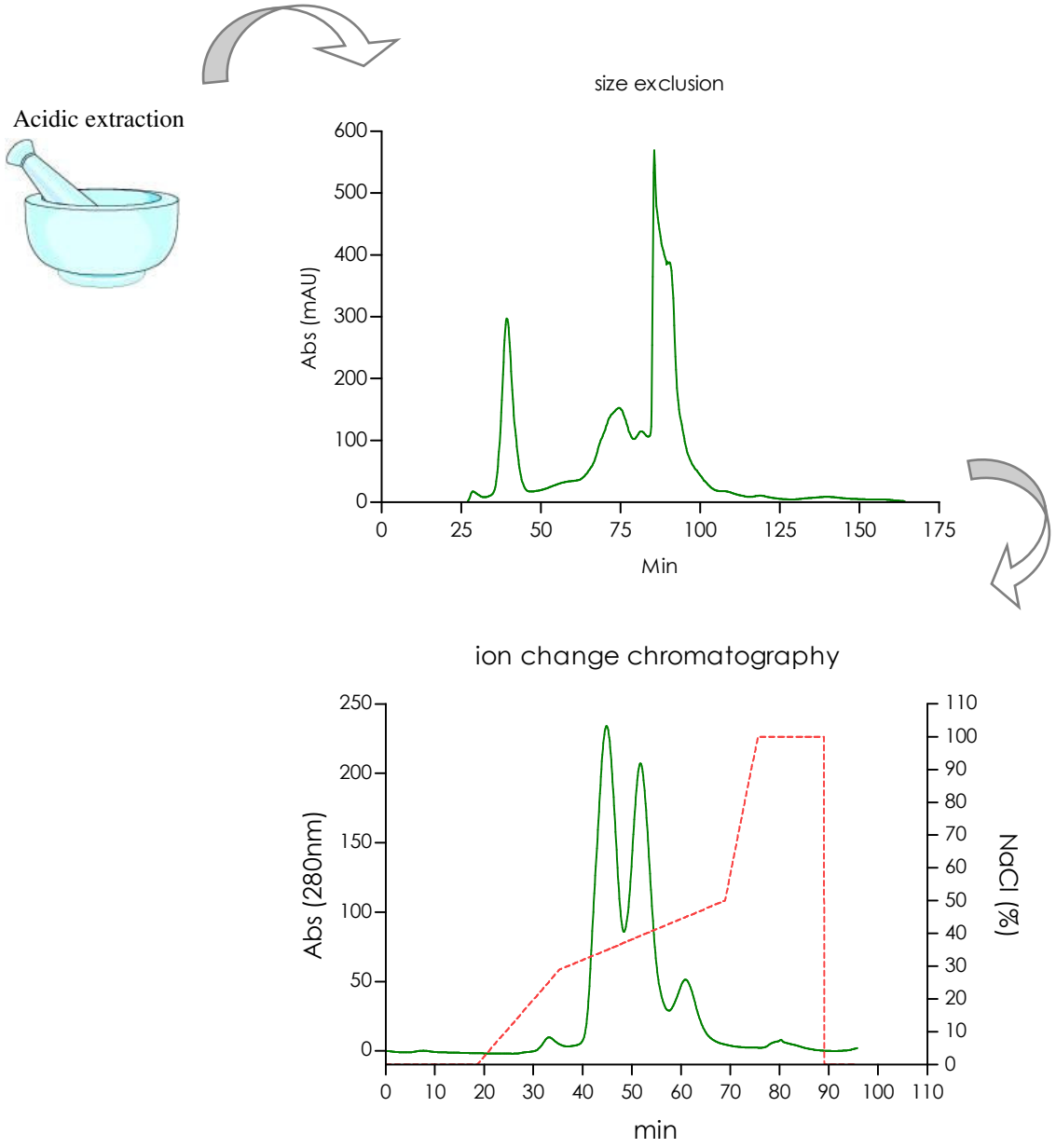


Figure 1.8 – Simple representation of cardosins purification protocol. The red arrow in size exclusion chromatogram identifies the fraction corresponding to cardosins mixture that is subsequently applied to ion change resin to obtain cardosins A_0, A and B.

Furthermore, cardosins account for more than 60% of the total protein content in mature stigmas and are the first example of extremely abundant proteinases in higher plants. In this way, together they account for the majority of the soluble protein in mature pistils, which allows their purification in a large scale.

[137] Pereira AO, Cartucho D, Duarte AS, Gil MH, Cabrita AS, Patrício, JÁ, Barros MT. Immobilisation of cardosin A in chitosan sponges as a novel Implant for drug delivery. Current Drug Discovery Technologies, Article in Press.

The chromatographic fraction signalled by a red arrow in Fig. 1.8, compromise the three complementary activities from *C. carduncullus* L. acidic extract that could be used as an inexpensive and well defined enzymatic tool for several applications. In addition to the characteristics mentioned above, namely in what concerns their specificity, this feature supports the implementation of a new protocol for the establishment of primary cultures of neurons that uses cardosins as enzymes for cell disaggregation.

As cited beyond collagen type IV, laminins vitronectin and fibronectin are typical components of brain extracellular matrix. Some sequences of these ECM proteins were analysed with regard to the known primary specificity for cardosins and several hypothetical cleavage sites were selected and compared with the cleavage of specific peptide bonds for trypsin (Appendix, Fig. 7.1-7.5, for sequence examination). Even though knowing that tertiary and quaternary structure of this proteins might be hiding the selected peptide bondes, a straightforward observation of their sequences reveal that there are less target peptide bonds for cardosins then for trypsin, which could be particularly useful for cell integrity preservation during brain disaggregation with cardosins.

Since, for cell isolation there is a need to cut cell-cell and cell-ECM interactions minimizing damage to individual cells, it is auspicious to take advantage from the narrow specificity of these proteolytic enzymes.

2. Aims

2. Aims of the present study

The main objective of this research is to evaluate cell behavior after isolation from brain tissue using cardosins in place of trypsin for brain disaggregation.

After neuronal primary cultures establishment from rat cortical brain, neuronal viability of the cultured cells over a 7 days period will be assessed.
To accomplish this task, it will be adopted a multi-modal approach, that includes the traditional methodologies for viability determination (MTT reduction assay & Tripan Blue staining) combined with other strategies. These comprise, for instance, morphological evaluation by immunocytochemistry and the employment of molecular methods to determine protein expression.

The scheme described beyond, will be further used to evaluate cell recover after mechanical lesion in neuronal cultures, mainly, regarding neuritogenesis re-expression.

In addition, in the frame of this study, it will be particularly interesting to establish a relationship between neuronal development and MMPs expression, given that these ECM-degrading proteases, play important roles in the cellular response to several environmental stimulus.

3. ESTABLISHMENT OF NEURONAL PRIMARY CULTURES

3. Establishment of neuronal primary cultures

3.1. Brief introduction

The properties of individual cells are difficult to study in the context of a multi-cellular organism. To avoid this difficulty, researchers study cells growing in culture. The two major advantages are the control of physiochemical environment (pH, temperature, osmotic pressure, O_2 and CO_2 tension), which may be controlled very precisely, and the physiological conditions, that may be kept relatively constant but cannot always be defined. Recent progress in the biology of cell adhesion is enabling cell culture models to better reproduce *in vivo* functions, allowing a better knowledge of the molecules involved in adhesion to extracellular matrix and neighbouring cells that are important regulators of cell behaviours[138]. One of the advantages of this technique is that although the number of cells obtained from small sample pieces of tissue is little, it can be increased in a short period of time[139]. The homogeneity of the sample is undertaken and in terms of pharmacological/toxicological investigation, less compound quantity is needed when compared to quantity required for animal model experiments.

Another benefit of cell culture resides on the possibility of cryopreservation, which leads to reduce the risk of microbial contamination and morphological changes. Furthermore, work can be conducted using cells at a consistent passage number.

Thus, the advantages of cell culture are fundamentally resumed in 4 topics: i) the cellular environment can be manipulated; ii) the cell type can be well defined; iii) the cells can be obtained in large quantities; and iv) many different cellular phenomena can be investigated. These include growth, differentiation, locomotion, as well as the responses to hormones, drugs or carcinogens.

The development of tissue and cell cultures owes, mainly, to the needs of medical research, such as the production of antiviral vaccines and the

[138] Bhadriraju K, Chen CS (2002). Engineering cellular microenvironments to improve cell-based drug testing. Drug Disc Tod. 7:612-620.

[139] Yildiz, C; Bahçe, M; Deveci, S; Bilgili, H; Ide, T; Tunay, S; Basbozkurt, M; Gur, E. 2001. Autologous chondrocyte transplantation (An experimental study in rabbit knee joints). Arthroplasty Arthroscopic Surgery. 12(1): 75-82.

understanding of neoplasia. An additional force of increasing pressure from public opinion has been the expression of concern by many animal-rights groups over the unnecessary of experimental animals. The introduction of more sensitive and more readily performed *in vitro* techniques, together with a very real prospect of assaying for inflammation *in vitro*, has promoted an extraordinary expansion of *in vitro* testing. In addition to cancer research and virology, other areas of investigation have become dependent on tissue culture techniques. The insight on the mechanism of action of antibodies, derived from monoclonal antibody techniques was, like the technique of cell fusion, a preamble of a new field of investigation based on genetic manipulation. Other areas of major interest comprise the study of cell interactions and intracellular control mechanisms in cell differentiation and development. Based on cell culture methodology, neurological research has greatly progressed on the last few years, contributing to disclose some of numerous mechanisms that role nervous function.

Tissue culture technology has also been used for many routine medical or industrial applications. For instance, chromosomal analysis of cells derived from the amniotic fluid by amniocentesis can reveal genetic disorders in the unborn child[140,141]; viral infections can be tested qualitatively and quantitatively on monolayers of appropriate host cells[142,143], and the toxicity of pharmaceutical compounds[144,145], as well as, potential environmental pollutants may be measured in colony-forming and other *in vitro* assays[146]. Further developments in the application of tissue culture to medical problems may follow from the demonstration that cultures of epidermal cells form functionally differentiated sheets in culture[147] and endothelial cells may form capillaries[148], suggesting

[140] Bell JA, Pearn JH, Smith A (1987). Prenatal cytogenetic diagnosis. Amniotic cell culture versus chorionic villus sampling. Med J Aust., 5;146(1):27-9. Review.
[141] Levade T, Enders H, Schliephacke M, Harzer K (1995). A family with combined Farber and Sandhoff, isolated Sandhoff and isolated fetal Farber disease: postnatal exclusion and prenatal diagnosis of Farber disease using lipid loading tests on intact cultured cells. Eur J Pediatr. 154(8):643-8. Review.
[142] Kesel AJ (2005). Synthesis of novel test compounds for antiviral chemotherapy of severe acute respiratory syndrome (SARS). Curr Med Chem.12(18):2095-162. Review.
[143] Ray AS (2005). Intracellular interactions between nucleos(t)ide inhibitors of HIV reverse transcriptase. AIDS Rev. 7(2):113-25. Review.
[144] Hornof M, Toropainen E, Urtti A (2005). Cell culture models of the ocular barriers. Eur J Pharm Biopharm. 60(2):207-25. Review.
[145] Forbes B, Ehrhardt C (2005). Human respiratory epithelial cell culture for drug delivery applications. Eur J Pharm Biopharm. 60(2):193-205. Epub 2005 Apr 21. Review.
[146] Aufderheide M (2005). Direct exposure methods for testing native atmospheres. Exp Toxicol Pathol. 57 Suppl 1:213-26. Review.
[147] Eming SA, Smola H, Krieg T (2002). Treatment of chronic wounds: state of the art and future concepts. Cells Tissues Organs. 172(2):105-17. Review.

possibilities in autografting and reconstructive surgery using individual's own cells[149,150,151]. The prospects for implantation of normal cells from adult or fetal tissue-compatible donors or genetically reconstituted cells from the same patient, are now very actual[149,150,148]. The technical barriers are progressively being overcome. The practical feasibility of normal fetal neuron implantation into patients with Parkinson's disease has been demonstrated[152,153]. There is newly evidence that cell culture allow autotransplantation of neuronal cells[154,155].

As it was mentioned previously, there are three varieties of cultured cells - primary cultured cells, cell strains and cell lines – all important tools to improve neurological discovery. Neuron isolation and the establishment of primary cultures offer several advantages for studies outside the complexity of the brain. First, enriched populations of neurons decrease the ambiguity of interpreting the cellular target of various test substances. Second, isolated neurons in culture allow direct access for application of pharmacological agents and electrophysiological recording. Third, cell culture allows precise control of the environment affecting the cell. These uniform conditions promote greater reproducibility in physiological measurements than whole animal studies. Fourth, cell culture allows continuous visual access for studies of morphological characteristics such as neuritogenesis, connectivity and toxicity[156]. The ability to maintain nerve and glial cells in primary cultures is essential for the advancement in the understanding of the developing nervous system. By using cell cultures, it has become possible to investigate cell-cell interactions that are difficult to study *in vivo*. However, the extent to which such

[148] Sieminski AL, Hebbel RP, Gooch KJ (2004). The relative magnitudes of endothelial force generation and matrix stiffness modulate capillary morphogenesis in vitro. Exp Cell Res. 297(2):574-84.
[149] Carella AM, Beltrami G, Corsetti MT (2003). Autografting in chronic myeloid leukemia. Semin Hematol. 40(1):72-8. Review.
[150] Ehrlich HP (2004). Understanding experimental biology of skin equivalent: from laboratory to clinical use in patients with burns and chronic wounds. Am J Surg. 187(5A):29S-33S. Review.
[151] Amoh Y, Li L, Campillo R, Kawahara K, Katsuoka K, Penman S, Hoffman RM. (2005). Implanted hair follicle stem cells form Schwann cells that support repair of severed peripheral nerves. Proc Natl Acad Sci U S A. 102(49):17734-17738.
[152] Boonman Z, Isacson O (1999). Apoptosis in Neuronal Development and Transplantation: Role of Caspases and Trophic Factors Experimental Neurology,156(1): 1-15.
[153] Helt CE, Hoernig GR, Albeck DS, Gerhardt GA, Ickes B, Reyland ME, Quissell DO, Stromberg I, Granholm AC (2001). Neuroprotection of grafted neurons with a GDNF/caspase inhibitor cocktail. Exp Neurol. Aug;170(2):258-69.
[154] Lee KH, Yoon do H, Park YG, Lee BH (2005). Effects of glial transplantation on functional recovery following acute spinal cord injury. J Neurotrauma, ;22(5):575-89.
[155] Klein S, Svendsen C (2005). Stem cells in the injured spinal cord: reducing the pain and increasing the gain. Nature Neuroscience 8, 259 – 260.
[156] Brewer G (1997). Isolation and culture of adult rat hippocampal neurons. Journal of Neuroscience Methods 71 (1997) 143–155.

cell cultures actually mimic the processes *in situ* is uncertain and difficult to assess. Cells may behave differently *in vitro* because of factors such as differences in the enzymes used for cell dissociation, in culture medium, matrices used, and soluble factors added as well as changes in the environment and the absence of other cells normally present in the tissue from which the cells are derived. Mixed cultures are therefore preferable, because all the cells present *in situ* are also present in vitro[157].

Taking advantage of cardosins hydrolytic features, described in more detail in the last chapter, the present work aims the implementation of a new protocol for the establishment of primary neuronal cultures by using cardosins instead of trypsin. In this chapter it is described the isolation method, as well as, the different kind of assays, usually used to assess cell viability of cultured neurons.

[157] Svenningsen A, Shan W-S, Colman DR, Pedraza L (2003). Rapid Method for Culturing Embryonic Neuron–Glial Cell Cocultures. Journal of Neuroscience Research 72:565–573.

3.2. Material & Methods

3.2.1 Reagents

Neurobasal medium, B27 supplement and trypsin (USP grade) were purchased from GIBCO. Mouse anti-MAP-2, rabbit-GFAP, glutamine, DNase (DN-25), hydrogen peroxide and trypan blue were purchased from Sigma Chemical Co. Goat anti-mouse IgG antibody conjugated to Alexa Fluor® 594 and goat anti-rabbit IgG antibody conjugated to Alexa Fluor® 488 were purchased from Molecular Probes. All other reagents were from Sigma Chemical Co. or from Merck KGaA.

3.2.2 Purification of cardosins

Cardosins were purified according to Sarmento and colleagues[158], by a method that allows the purification of high amounts of protein.
Briefly, stigmas from fresh flowers were homogenised in a mortar and pestle in sodium citrate 100 mM, pH 3.5, and centrifuged. The supernatant was applied to a Hiload Superdex 75 semi prep (Amersham Pharmacia Biotech) equilibrated and eluted with 25 mM Tris–HCl pH 7.6 at a flow rate of 3 ml/min.
The cardosins fraction obtained by size-exclusion chromatography is not further purified and, thus, compromise proteolytic activities from cardosins A_0, A and B. Purity of cardosins was assessed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli[159] after staining with Coomassie Brilliant Blue. Cardosins fractions were quantified and concentrated by lyophilization. Dried protein was stored at −80 ◦C (appendix: protocol 1, for details).
Periodically, cardosins activity was checked by an established method based on the hydrolysis of the synthetic peptide Lys-Pro-Ala-Glu-Phe-Phe(NO_2)-Ala-Leu (appendix: protocol 2).

[158] Sarmento AC, Oliveira CS, Pires EM, Amado F, Barros M (2004). Reverse hydrolysis by cardosin A. Specificity considerations. J Mol. Catal B: Enzym. 28: 33-37.
[159] Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

3.2.3 Cell isolation and culture

Primary cultures of brain cortical cells were prepared from Wistar rat embryos (E15-E16), as described by Agostinho and co-workers[160] with minor modifications (Fig. 3.1).

Animals were handled following the approved guidelines of national ethical requirements for animal research and with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. Briefly, pregnant females were sacrificed by cervical dislocation, the uterus removed under sterile conditions and the embryos dissected on a 100mm Petri dish with cold Ca^{2+}- and Mg^{2+}-free Hank's Balanced Salt Solution (CMF-HBSS), pH 7.4. The cortecis were carefully removed and placed in CMF-HBSS containing 0.3% of BSA in a 35mm Petri dish for subsequent removal of the meninges. After slightly minced, the cortecis were digested with 0.1% trypsin or 0.1% cardosins in CMF-HBSS containing 0.008% DNAse I, for 10 minutes at 37°C. Cardosins concentration was optimised in order to accomplish equivalent cell viability for both types of culture. The digestion was stopped by adding 10% fetal calf serum (FCS) and washing.

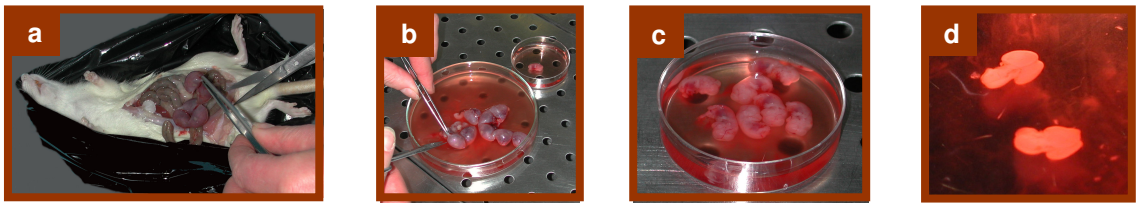


Figure 3.1 – Primary culture establishment: (a) sacrificed pregnant rat, (b) removal of the embryos (E15-16) from the uterus, (c) cortex dissection and (d) removal of the meninges. (These photographs were kindly provided by N. Rosa).

The digested tissue was mechanically dissociated by gentle forcing through a 5 ml glass pipette. After centrifugation (140 x g, 5 minutes), the cells were resuspended in Neurobasal Medium supplemented with 2% B27, 0.5mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin. Cell viability was

[160] Agostinho P, Oliveira CR (2003). Involvement of calcineurin in the neurotoxic effects induced by beta-amyloid and prion peptides. Eur. J. Neurosci. 17: 1189-1196.

assessed by Tripan blue exclusion and counting on a hemocytometer. The cells were plated on poly-D-lysine (0.1 mg/ml) coated multi-well plates, at a density of $0.5x10^6$ cells/cm^2, or 10 mm glass coverslips at a density of $0.25x10^6$ cells/cm^2. The cells were maintained at 37°C in a humidified 5% CO_2/95% air atmosphere.

3.2.4 MTT assay of cell viability

The viability of cultured cells was quantitatively assessed by the MTT (3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyl-tetrazolium bromide) reduction test. Living cells convert MTT to a dark blue, water-insoluble formazan product formed by the reduction of the tetrazolium ring of MTT by the mitochondrial succinate dehydrogenase[161].

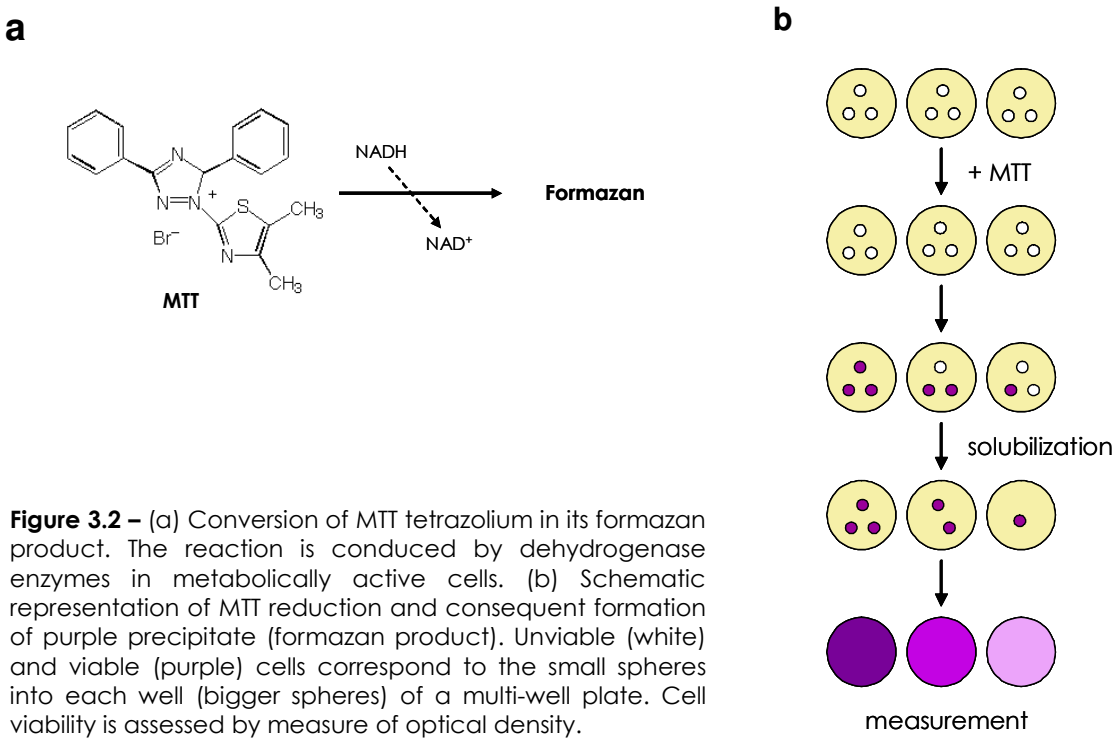


Figure 3.2 – (a) Conversion of MTT tetrazolium in its formazan product. The reaction is conduced by dehydrogenase enzymes in metabolically active cells. (b) Schematic representation of MTT reduction and consequent formation of purple precipitate (formazan product). Unviable (white) and viable (purple) cells correspond to the small spheres into each well (bigger spheres) of a multi-well plate. Cell viability is assessed by measure of optical density.

[161] Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63

As it is represented in Fig. 3.2, colour intensity is directly proportional to cell dehydrogenase activity and consequently indicates de ratio of viable cell in the cultures. The cultures were incubated with 300µl of MTT solution (0.5mg/ml in Krebs buffer) at 37°C for 2 hours. After washing, the cultures were incubated with 0.08N HCl in isopropanol to dissolve the blue formazan product, and the optical density read at 570nm in a microplate reader with background subtraction at 620nm. Results were expressed as percentage of the optical density in controls.

3.2.5 Cell Survival Assays

Glutamate excitotoxicity: The optimum glutamate concentration for starting cultures of embryonic neurons is 25 µM (Brewer *et al*., 1993[162]). This relatively high glutamate concentration may be a useful mimic of the leaky blood brain barrier of embryos. Therefore, survival of neurons isolated with cardosins versus trypsin was evaluated with and without glutamate in the culture medium. Cell survival was determined 24 hours after by using the MTT assay.

Cell response to H_2O_2: 7 days old cultures were treated without or with H_2O_2 (10 and 50 µm). Cell survival was determined 24 hours after by using the MTT assay.

3.2.6 Immunocytochemistry

Cell identity and morphology were evaluated after the immunocytochemical labeling of the cortical neurons with an anti-MAP-2 (microtubule-associated protein 2) antibody, and for the astrocytes with an anti-GFAP (glial fibrillary acidic protein) antibody. After removal of the culture medium and washing with phosphate buffered saline (PBS), the cells were fixed with 4% paraformaldehyde in PBS for 10 min, followed by permeabilization with 0.2% Triton X-100 in PBS for 10 min. After blocking for 90 min with 0.2% gelatin in PBS,

[162] Brewer, G.J., Torricelli, J.R., Evege, E.K. and Price, P.J. (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free combination, J. Neurosci. Res., 35: 567–576.

the cells were incubated with the anti-MAP-2 (1:500) and anti-GFAP (1:200) antibodies for 90 min. After extensive washes in PBS, the cells were incubated for 60 min with the secondary antibodies: anti-mouse IgG conjugated to Alexa Fluor 594 (2 μg/ml) and anti-rabbit IgG conjugated to Alexa Fluor 498 (1 μg/ml). To assess the specificity of the immunostaining, the primary antibodies were omitted in some coverslips. Finally, the coverslips were washed thoroughly and mounted on glass slides in fluorescent mounting medium (DAKO). The detailed experimental proceeding is available in the appendix section, as protocol 3. The preparations were observed in a Carl Zeiss fluorescence microscope, and images were acquired with the AxioVision software (Carl Zeiss Imaging Systems).

3.2.7 Immunocytochemical and morphometric analysis of cell cultures

In order to obtain data about neurite outgrowth and neuronal sprouting after brain disaggregation using cardosins, the number of neurites and their length were evaluated in four randomised areas of each experiment, in three independent preparations. All analyses were developed for cultures established with trypsin for further comparison. The results were acquired by using the AxioVision software, followed by statistical evaluation with Student's t-test.

3.2.8 Data analysis

Data are expressed as means ± SEM. Statistical significance was determined by using Student's t-tests, *$p \leq 0.05$, **$p \leq 0.001$ and ***$p \leq 0.001$, for significant, very significant and extremely significant, respectively, as indicated in the figure legends.

3.3. Results & Discussion

3.3.1 Cell yield and viability

To study the feasibility of using cardosins to prepare neuronal cell cultures, its ability to dissociate cortical tissue from rat embryonic brain was determined, and it was compared the cell yield and the cell viability with those of an established protocol to prepare cortical cultures using trypsin[160]. The cell yield of cortex dissociation with increasing concentrations of cardosins mixture was previous determined[163,164]. The optimal concentration of cardosins for the isolation of cortical cells was 1mg/ml since the cell yield at this concentration was similar to that obtained with trypsin; the use of higher concentrations of cardosins did not further increase the cell yield. Cortex dissociation with 1 mg/ml cardosins yielded a number of cells, approximately $20x10^6$ cells/mg wet tissue, similar to the yield obtained with trypsin at the concentration routinely used in the laboratory to isolate cortical cells from rat embryos. Immediately after cell isolation, cell viability was assessed by trypan blue exclusion method (Fig. 3.3).

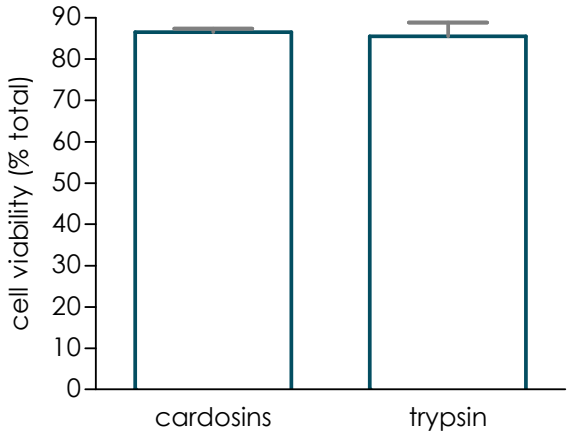


Figure 3.3 – Cell viability of isolated neurons assessed by Tripan blue method.

[163] Rosa N (2002). Utilização de uma proteinase aspártica vegetal no isolamento de neurónios do córtex cerebral de rato. Tese de mestrado. Universidade de Coimbra, Portugal.
[164] Duarte AS, Rosa N, Duarte EP, Pires E, Barros MT. Cardosins: a new and efficient plant enzymatic tool to isolate neuronal cells for culture. Journal of Neuroscience Methods (*submitted*).

The percentage of viable cells isolated with cardosins (86.49 % ± 0.81 of total) was quite similar to the viability of cells dissociated with trypsin (85.56 % ± 3.30). Significantly, the viability of cells obtained with cardosins was less variable than those obtained with trypsin.

Cell viability was also examined upon culturing the cells (Fig. 3.4). Using the MTT reduction assay, it was possible to achieve similar viabilities for cells isolated with cardosins or with trypsin 3 hours after plating, but at 24 h the viability of cells that have been isolated with cardosins was much higher than the viability of cells isolated with trypsin ($p \leq 0.001$). The difference in viability was no longer significant upon 7 days in culture. The increase in cell viability observed upon culturing is due to cell recovery after the isolation and to the death and detachment of nonviable cells, leading to the increase in the percentage of cells capable of reducing MTT. The faster increase in cell viability observed for the cells isolated with cardosins as compared to cells isolated with trypsin suggests that cardosins are less harmful to neuronal cells.

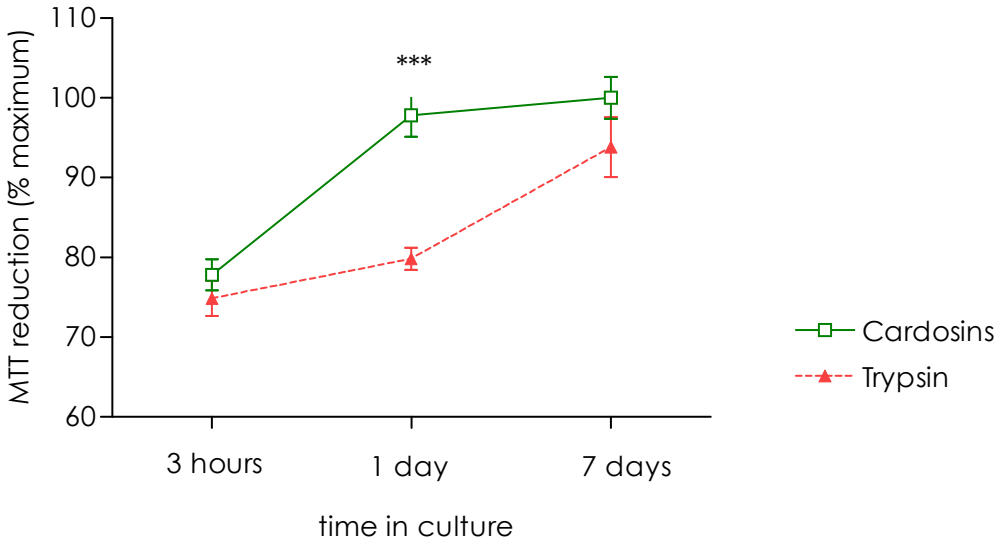


Figure 3.4 – Cell viability of cultured neurons for 7 days measured by MTT reduction assay.

3.3.2. Immunocytochemical and morphometric analysis of cell cultures

In order to identify the cells present in the cultures and to follow their development the cultures were immunolabeled for a neuron specific protein, the microtubule-associated protein-2 (MAP-2), and for a glial cell marker, the glial fibrilary acidic protein (GFAP). The immunofluorescence images shown in Fig. 3.5 evidenced that neurons isolated with cardosins and subsequently cultured exhibited a normal morphology, with neurites extending from the cell bodies, demonstrating a normal cellular growth, as a result of re-expression of neuritogenesis, apparently without damage. Actually, longer neurites were observed in cultures prepared with cardosins, 24 hours after plating, as compared to the cultures of cells dissociated with trypsin (Fig. 3.5 a,b) but the differences were no longer apparent in cells cultured for longer periods (Fig. 3.5 c,d). Immunostaining for GFAP showed the presence of astrocytes in both cultures (Fig. 3.5 e,f), which is not unexpected since the Neurobasal medium with the B27 supplement does not hinder the proliferation of nonneuronal cells. Since the growth of astrocytes appear to be similar in both cell cultures, the differences in the neuronal development cannot be accounted for by differences in astrocyte support.

Quantitative analysis showed that neurons isolated with cardosins exhibited larger numbers and longer neurites (Fig. 3.6). The number of neurites extending longer than 25 μm was 68.0 ± 5.0 in cells that had been isolated using cardosins, which is significantly larger than in trypsin isolated cells (39.5.0 ± 4.0, $p \leq 0.01$). Their maximal length was 35.0 μm ± 4.0 for trypsin generated cultures, and 50 μm ± 6.0 ($p \leq 0.01$) for cultures obtained with cardosins. No differences were perceptible after 7 days in culture. These morphological data are in agreement with cell viability data shown in Fig. 3.5.

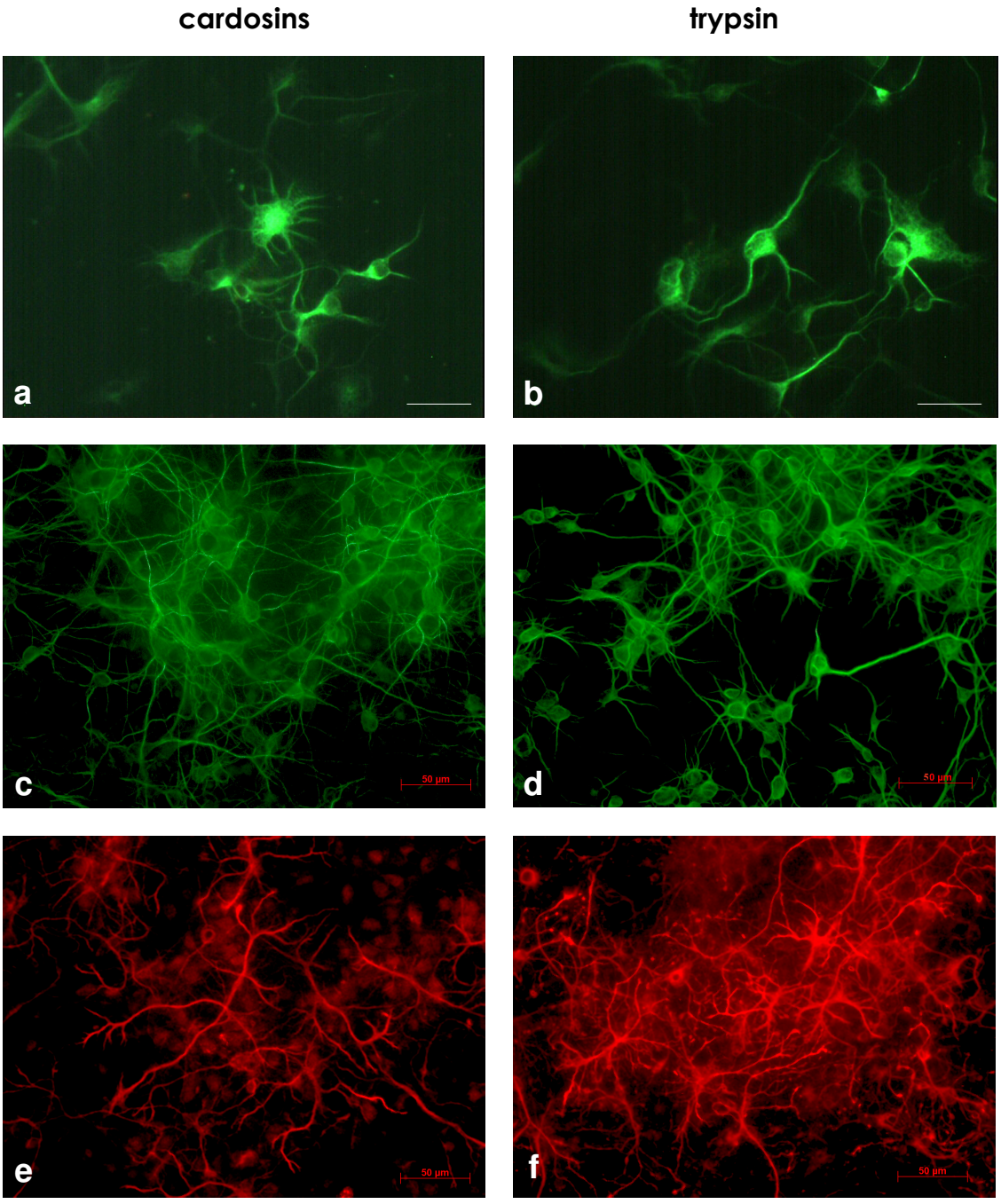


Figure 3.5- Immunocytochemical characterization of cell cultures prepared by cardosins or trypsin dissociation of embryonic brain cortex. The cells were cultured for 1 day (**a, b**), or for 7 days (**c-f**) before fixation and immunolabeling for MAP-2 (**a-d**), a neuronal marker, and for GFAP (**e, f**), a glial marker. Fluorescence microphotographies **c** and **e**, and **d** and **f** are from the same fields. (Scale bar = 50µm.)

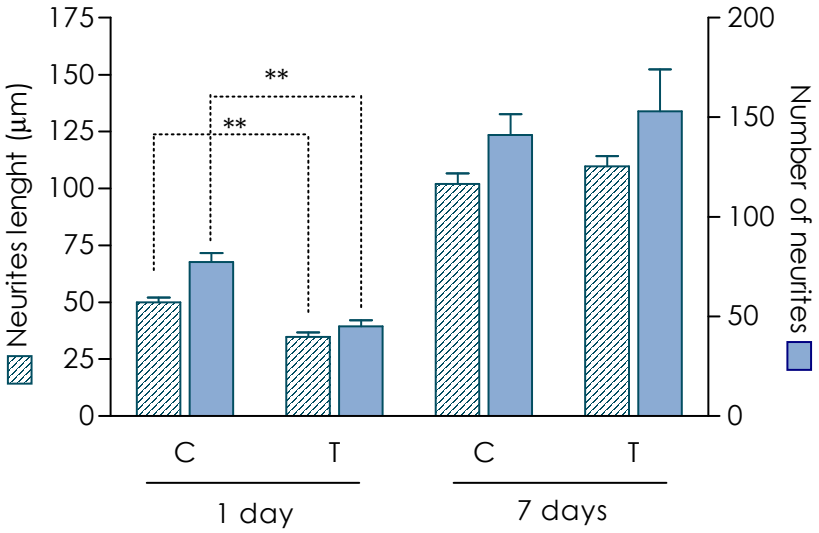


Figure 3.6 - Morphometric analysis of neurons cultured for 1 or 7 days after cell isolation with cardosins (C) or trypsin (T). The cultures were immunostained for MAP-2, and the neurite length and the number of neurites extending longer then 25 µm were determined in 4 randomly chosen fields. Data shown are the means ± SEM of determinations in 3 independent preparations.

Synapse is the means by which an impulse is transmitted between neural cells[165]. The axon of one neuron synapses with the dendrites of other neurons. The many-branched, terminal portion of an axon not only may synapse with many other neurons but also may synapse with numerous points on a single neuron. Each terminal branch of an axon ends in a synaptic bouton. A gap called the synaptic cleft separates the presynaptic membrane of a synaptic bouton from the postsynaptic membrane of a dendrite, or a non-nervous target cell, such as a muscle cell. Transmission across a cleft is accomplished by the diffusion of a chemical transmitter released by synaptic vesicles in the bouton of the axon. Impulses travelling down the length of the axon of the presynaptic neuron change the permeability of the bouton membrane allowing Ca^{2+} ions to diffuse into the bouton from the surrounding fluid. The presence of Ca^{2+} causes the synaptic vesicles to fuse with the presynaptic membrane and rupture releasing the transmitter chemical into the cleft. The transmitter chemical, such as acetylcholine, binds weakly with the postsynaptic membrane. This action alters the potential of the membrane and gives rise to a

[165] Nakanishi S (2005). Synaptic mechanisms of the cerebellar cortical network. Trends Neurosci 28(2):93-100. Review

new impulse in the postsynaptic neuron[166]. Immunocytochemistry allows, not only assessing to the cell morphology but, in the some cases, may reveal cell functionality. Thus, new synaptic boutons formation reveals the state of synapse development and plasticity.

An apparent rearrangement of microtubule loop architecture occurs during bouton division. The control of microtubule organization by MAPs may represent a common mechanism for regulated growth cone motility as well as synaptic growth and plasticity[167]. In this sense, immunocytochemistry was used to calculate the number of synaptic boutons.

The graph represented in the Fig. 3.7 is the result of statistical treatment of data obtained by counting the referred fluorescent points (correspondent to the synaptic boutons) in MAP-2 immunolabelled preparations of cortical neurons. The results – 182 ± 15 and 180 ± 18 for cardosins and trypsin, respectively - indicated that both neuronal cultures, 7 days after brain disaggregation, have the same synaptic plasticity evidencing normal growth-cone motility. This is further confirmed by the equivalent pathfinding shown by the developed neurons isolated with cardosins or with trypsin. Fig 3.8 shows representative images of cultures established by cardosins and trypsin after 7 days, where it is possible to visualise the synatic boutons through the neurite network.

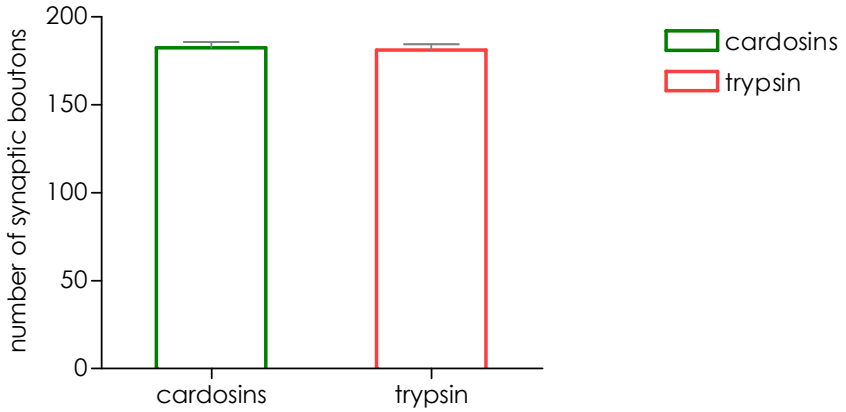


Figure 3.7 – Comparison of the number of synaptic boutons for the two types of cultures, after 7 days incubation period (determined in 4 randomly chosen fields). Data shown are the means ± SEM of determinations in 3 independent preparations.

[166] Purves D, Augustine G, Fitzpatrick D, Katz L, Lamantia A-S, McNamara J, Williams S. Neural Signaling, chap I *in Neuroscience*, 2nd edition, 2001 by Sinauer Associates

[167] Roos J, Hummel T, Ng N, Klambt C, Davis GW (2000). Drosophila Futsch regulates synaptic microtubule organization and is necessary for synaptic growth. Neuron 26(2): 371-82.

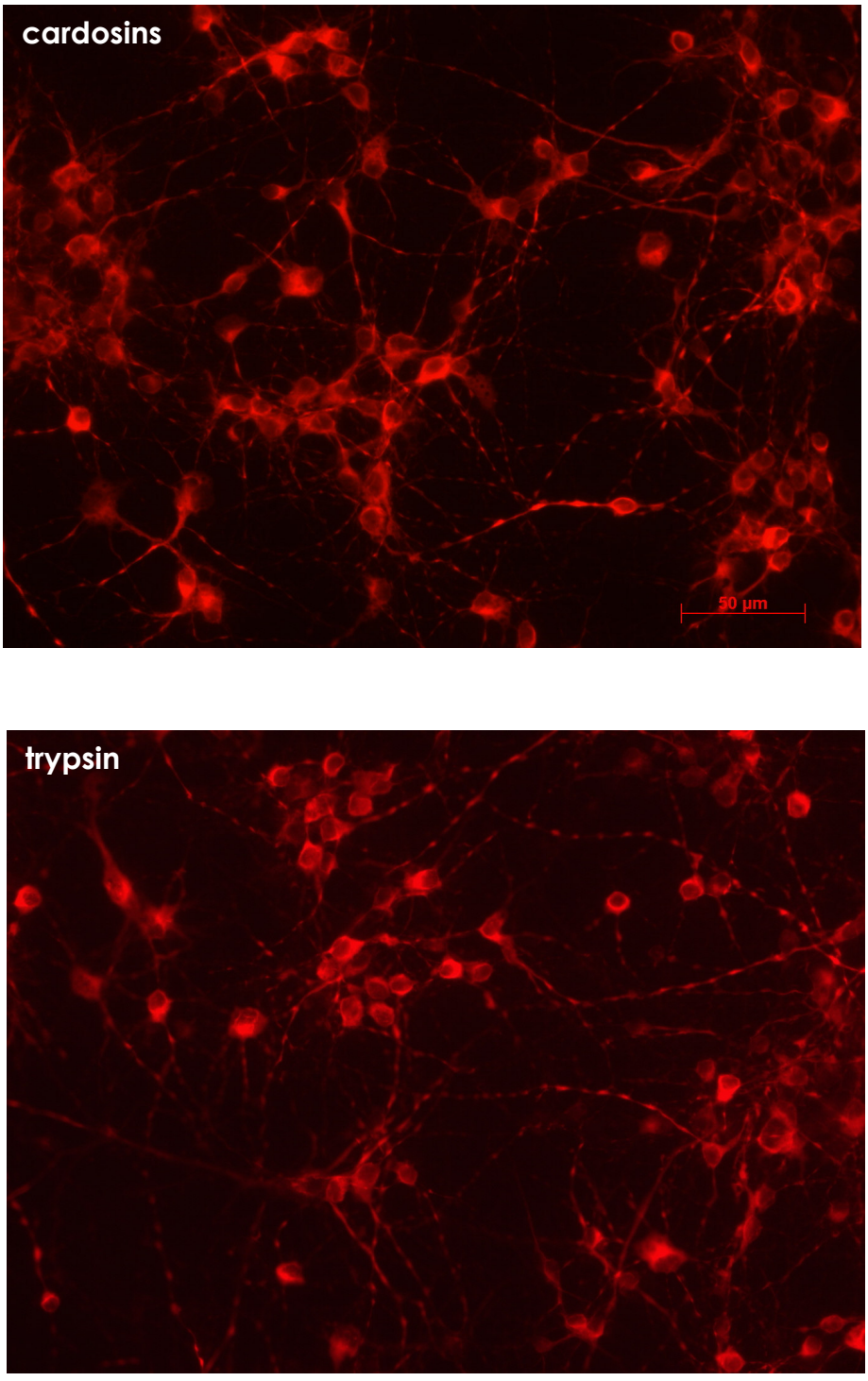


Figure 3.8 – Synaptic boutons. Representative photographs of cortical neurons obtained by using cardosins or trypsin in the isolation procedure (as described in the material & methods of this chapter).

3.3.3 Functional analysis of cell cultures

A preliminary study, based on neurotransmitter release in response to membrane depolarization, was performed by Rosa, 2002[163] and neuronal functionality after using cardosins for brain disaggregation was verified. The neurotransmitter GABA was chosen because of its abundance in the cortex and because its release from cultured cells is well characterized[168]. GABA release in the absence of stimulation was quite high, about 15% of the cell content, but similar in cardosins- and trypsin-prepared cultures. This elevated release of neurotransmitters in the absence of stimulation is often observed in embryonic cultures due to high levels of spontaneous activity[169]. Upon depolarization induced by increasing K^+ concentration in the extracellular medium, an additional release of GABA was observed, about 5% of the cell content, showing that the cells were capable of responding to a depolarizing stimulus, as expected from cells keeping a membrane potential.

To determine whether the cell cultures prepared with cardosins exhibit physiological responses several tests were developed.

A number of studies have indicated that glutamate can play an important role in developmental plasticity. The speed and direction of growth of cultured neurites is influenced by the application of glutamate[170].

A significant part of this modulation of neuritic outgrowth may depend on glutamate receptor mediated changes in intracellular Ca^{2+}.

Synaptogenesis and axonal branching are also influenced by glutamate receptors, as studied in retina ganglion cells, cerebellum, somatosensory cortex and superior colliculus. Migration of neurons during early development is regulated by glutamate, as shown in cerebellar granule cells. Glutamate, to a large degree acting through Ca^{2+}, also plays a critical role in modulating gene expression. However, high levels of extracellular glutamate inhibit the import of cystine, resulting in the depletion of glutathione and a form of cell injury called

[168] Santos PF, Carvalho AL, Carvalho AP, Duarte CB (1998). Differential acetylcholine and GABA release from cultured chick retina cells. Eur. J. Neurosci. 10: 2723-2730.
[169] Van Den Pol AN, Gao X-B, Patrylo PR, Ghosh PK, Obrietan K (1998). Glutamate Inhibits GABA Excitatory Activity in Developing Neurons. J. Neurosci. 18: 10749 10761.
[170] Van Den Pol A, Obrietan K, Cao V, Trombley P (1995). Embryonic hypothalamic expression of functional glutamate receptors Neuroscience, 67(2): 419-439

oxidative glutamate toxicity. For this reason, glutamate is extensively used in excitotoxicity studies.

The physiological consequences of extracellular glutamate are mediated by three classes of membrane proteins within the CNS. These are ionotropic glutamate (iGlu) receptors, metabotropic glutamate (mGlu) receptors (receptor superfamily that includes GABA receptor), and the cystine/glutamate antiporter. They are responsible for the majority of excitatory neurotransmission and also for a great deal of CNS pathology.

In cases of stroke or trauma, excessive extracellular glutamate leads to nerve cell death via the activation of NMDA receptors. This phenomenon, which can be reproduced in cell culture, is termed excitotoxicity[171].

Little is known on the expression and function of individual glutamate receptors during the prenatal development of the CNS. Recently, Di Giorgi Gerevini & colleagues[172] described the temporal profile of mGlu receptor expression in different brain regions from embryonic day 12 (E12) to the adulthood in relation to the sites of active neurogenesis. After glutamate receptors are expressed, neurons are susceptible to excitotoxicity from excess glutamate[173].

Based on these observations excitoxicity studies were carried out to assess neuronal physiology. Therefore, after 7 days of cultures establishment, survival of neurons isolated with cardosins versus trypsin was compared in culture medium with and without glutamate (Fig. 3.9). For neurons isolated with cardosins or trypsin, survival without glutamate was 34 or 52% better than with 25 μM glutamate, respectively (P<0.05).

Cultured cells were also examined to an additional toxic challenge (Fig. 3.10). Thus, after 7 days in culture, the cells were treated with H_2O_2, a reactive oxygen species known to increase oxidative stress and to cause neuronal death[174].

[171] Schubert D, Piasecki D (2001). Oxidative glutamate toxicity can be a component of the excitotoxicity cascade. J Neurosci., 21(19):7455-62.

[172] Di Giorgi Gerevini VD, Caruso A, Cappuccio I, Ricci Vitiani L, Romeo S, Della Rocca C, Gradini R, Melchiorri D, Nicoletti F (2004).The mGlu5 metabotropic glutamate receptor is expressed in zones of active neurogenesis of the embryonic and postnatal brain. Brain Res Dev Brain Res. May 19;150(1):17-22.

[173] Brewer, G.J., Torricelli, J.R., Evege, E.K. and Price, P.J. (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free combination, J. Neurosci. Res., 35: 567–576.

[174] Gorman AM, McGowan A, O'Neill C, Cotter T (1996). Oxidative stress and apoptosis in neurodegeneration (review). J. Neurol. Sci. 139: 45-52.

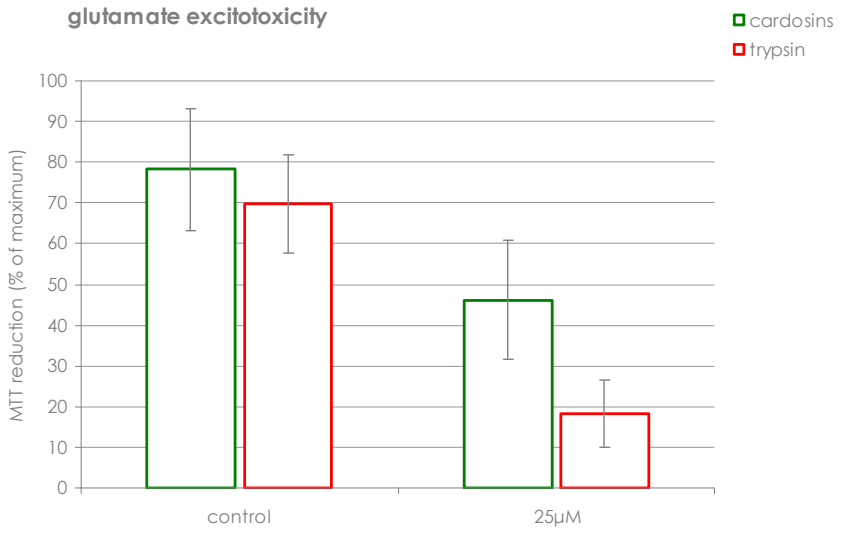


Figure 3.9 - Glutamate excitotoxicity. Cell cultures (7 days-old) were exposed to glutamate (25 µM) and cell viability was assessed, after a 24 h incubation period, with the MTT test. The results are presented as percentage of controls for trypsin and are expressed as means ± SEM of at least four independent experiments. *p≤0.05, as compared to cells isolated with trypsin.

Oxidative stress is believed to be implicated in a wide variety of human degenerative disorders of the CNS, including Alzeimer's disease, Parkinson´s disease and in pathological conditions such as ischemia[174,175176]. Rat embryonic neurons are particularly sensitive to H_2O_2 and the exposure of neuronal cultures to H_2O_2 induces a concentration-dependent (10-1000 µM) cell death by estimative after 24 hours[177].

As shown in Fig. 3.10, H_2O_2 caused a decrease in MTT reduction in both cultures, showing that neurons were responding in the same manner to the toxic insult. However, neurons obtained with cardosins showed higher cell viability both in control and in cultures treated with 10 µM H_2O_2 (p≤0.05), that could be a result of cellular integrity and consequent better coordination of all oxidative stress mechanisms.

[175] Facheris M, Beretta S, Ferrarese C (2004). Peripheral markers of oxidative stress and excitotoxicity in neurodegenerative disorders: tools for diagnosis and therapy? J. Alzheimers Dis. 6: 177-84.
[176] Mariani E, Polidori MC, Cherubini A, Mecocci P (2005). Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview. J Chromatogr B Analyt Technol Biomed Life Sci. 827(1): 65-75.
[177] Desagher S, Glowinski J, Premont J (1996). Astrocytes protect neurons from hydrogen peroxide toxicity. J. Neurosci. 16: 2553-2562.

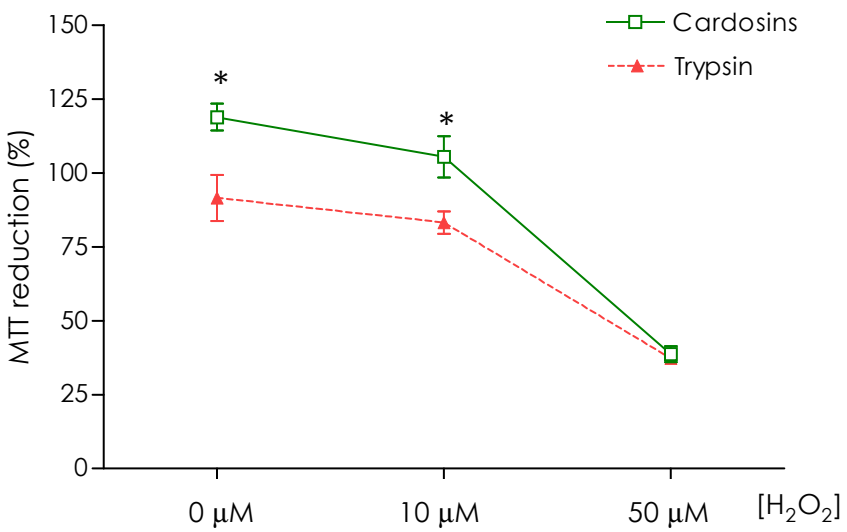


Figure 3.10 - Cell response to a toxic challenge - H_2O_2 effect. Cell cultures (7 days-old) were exposed to H_2O_2 (10 or 50 µM) and cell viability was assessed, after a 24 h incubation period, with the MTT test. The results are presented as percentage of controls for trypsin and are expressed as means ± SEM of at least four independent experiments. *p≤0.05, as compared to cells isolated with trypsin.

4. MMPS INVOLVEMENT IN THE MECHANISMS OF NEURONAL REGENERATION AND FUNCTIONAL RECOVERY

4. MMPs involvement in the mechanisms of neuronal regeneration and functional recovery

4.1 Brief introduction

In addition to their structural function, extracellular matrix (ECM) molecules play an important role in the structural rearrangements that accompany many physiologic, as well as pathologic, processes. To preserve tissue integrity during these processes, ECM synthesis and degradation must be exquisitely regulated. Cell-cell and cell-matrix interactions play critical roles in all phases of developmental tissue remodelling, where MMPs function in the degradation of a broad-spectrum of ECM and basement membrane proteins, including collagens, laminin, fibronectin, glycoproteins and proteoglycans.

Investigation on the MMPs' target molecules can help ascertain and explain MMPs' roles in brain physiology. Numerous substrates of potential physiological significance have been proposed as target molecules for theses matrix proteases. For example, *in vitro*, these enzymes cleave proteoglycans, including chondroitin sulfate proteoglycan (CSPG), that are abundant in the adult nervous system and evoke a strong avoidance reaction by a variety of neuronal subtypes.

Furthermore, MMPs have been considered as candidate molecules for enabling remyelination, because they can degrade all protein components of the ECM as demonstrated by McCawley and Matrisian, 2001[178].

Although MMPs are known to have detrimental roles after injury[179,180], their consistent upregulation in the damaged CNS invites the hypothesis that these proteases have important functions in the repair process, particularly in remyelination.

Preceding studies have shown that an increase in MMP-9 levels occurs during the period of developmental myelination in both the corpus callosum and the

[178] McCawley LJ, Matrisian LM (2001) Matrix metalloproteinases: they're not just for matrix anymore! Curr Opin Cell Biol 13: 534-540.
[179] Yong, VW, Krekoski, CA, Forsyth, PA, Bell, R, Edwards, DR (1998). Matrix metalloproteinases and diseases of the central nervous system. Trends Neurosci. 21: 75–80.
[180] Yong VW, Power C, Forsyth P, Edwards DR (2001) Metalloproteinases in biology and pathology of the nervous system. Nat Rev Neurosci 2: 502-511.

optic nerve[181,182]: the *in vitro* inhibition of MMP-9 reduced oligodendrocytes process extension. In addition, Larsen and co-workers in 2003[183] showed that mice lacking MMP-9 are impaired in myelin reformation after lysolecithin-induced demyelination. This deficiency may be explained at least in part by the failure to clear the accumulation of NG2 (a cell membrane-associated chondroitin sulphate proteoglycan present in nervous tissue cells that have not yet specialized into oligodendrocytes) an inhibitory proteoglycan that retards the maturation and differentiation of oligodendrocytes essential for remyelination. These results showed for the first time that some of the MMPs that are expressed after CNS injury have reparative functions, suggesting that the selective enhancement of these MMP activities may improve recovery from CNS insults.

As mentioned earlier, laminins, which are heterotrimers of α, β and γ chains, are target molecules of MMPs activity, representing one of the major components of all basal lamina in vertebrates and invertebrates. These glicoproteins play key roles in cellular mechanisms involving several signal paths. Mutations in laminin genes in humans and mice have demonstrated critical roles for laminins in diverse tissues, including muscle, kidney, lung, skin, and placenta[184]. Laminins possess considerable neuritepromoting activity with respect to primary cultured neurons taken from the mammalian embryonic brain. A role for laminin interacting with oligodendrocyte laminin-binding integrins in the formation of myelin membrane has been reported[185]. Purified laminins and laminin fragments affect cultured central neurons in numerous ways, including promotion of adhesion, migration, survival, and neurite outgrowth[186]. It has been stated that laminins are present in high amounts during periods of axonal growth and their expression levels diminish later in development[190,186]. Laminin immunoreactivity increases at locations of seizure-induced axonal sprouting, while facilitation of

[181] Uhm JH, Dooley NP, Oh LY, Yong VW (1998) Oligodendrocytes utilize a matrix metalloproteinase, MMP-9, to extend processes along an astrocyte extracellular matrix. Glia 22: 53-63.
[182] Oh LY, Larsen PH, Krekoski CA, Edwards DR, Donovan F, Werb Z, Yong VW (1999). Matrix metalloproteinase-9/Gelatinase B is required for process outgrowth by oligodendrocytes. *J. Neurosci.* 19, 8464–8475.
[183] Larsen PH, Wells JE, Stallcup WB, Opdenakker G, Yong VW (2003). Matrix metalloproteinase-9 facilitates remyelination in part by processing the inhibitory NG2 proteoglycan. J Neurosci. 23(35):11127-35.
[184] Yin Y, Kikkawa Y, Mudd JL, Skarnes WC, Sanes JR, Miner JH (2003). Expression of Laminin Chains by Central Neurons: Analysis With Gene and Protein Trapping Techniques. genesis 36:114–127.
[185] Buttery PC, Ffrech-Constant C (1999). Laminin-2/integrin interactions enhance myelin membrane formation of oligodendrocytes. Mol. Cell Neurosci. 14: 199–212.

laminin degradation in the hippocampus leads to accelerated neuronal cell death[187]. Laminins are also critical for formation and function of the peripheral nervous system, including migration of sympathetic and sensory neuroblasts, myelination of peripheral axons, and maturation of the skeletal neuromuscular junction[188]. Concerning this issue and using cultured dorsal root ganglionic (DRG) neurons, Zuo and co-workers (1998)[189] described that neuritic outgrowth was increased significantly by first treating the nerve sections with MMP-2, which repressed the inhibitory activity of CSPGs and unmasked the neurite-promoting activity of associated laminin. Parallel results were obtained for MT5-MMP (MMP-24)[190].

Finally, regarding MMPs' activity on growth factores, it is well established that neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) participate in neuronal survival and synaptic plasticity. It was recently reported that their precursors contain consensus sites for cleavage by MMP-3 and MMP-7[191]. Indeed, the proteases can extracellularly cleave secreted pro-neurotrophins[192].
Given the above findings it is reasonable to believe that imbalance between the normal levels of MMP activity promotes remodeling, and inhibition of their actions by TIMPs may result in abnormal development and consequently to a pathological state.

Hence, MMPs are crucial molecules for axonal guidance development and, therefore may facilitate cell migration to injured areas that require replenishment or may promote process extension as a prelude of cell interaction.

---

[186] Venstrom XA, Reichardt LF (1993). Extracellular Matrix 2: Role of extracellular matrix molecules and their receptors in the nervous system FASEBJ. 7: 996-1003.
[187] Wright JW, Harding JW (2004).The brain angiotensin system and extracellular matrix molecules in neural plasticity, learning, and memory. Prog Neurobiol. 2004 Mar;72(4):263-93. Review.
[188] Patton BL (2000). Laminins of the neuromuscular system. Microsc Res Tech 51:247–261.
[189] Zuo J, Ferguson TA, Hernandez YJ, Stetler-Stevenson WG, Muir D (1998) Neuronal matrix metalloproteinase-2 degrades and inactivates a neurite-inhibiting chondroitin sulfate proteoglycan. J Neurosci 18:5203-5211.
[190] Hayashita-Kinoh H, Kinoh H, Okada A, Komori K, Itoh Y, Chiba T, Kajita M, Yana I, Seiki M (2001). Membrane-type 5 matrix metalloproteinase is expressed in differentiated neurons and regulates axonal growth. Cell Growth Differ 12(11):573-80.
[191] Rattenholl A, Ruoppolo M, Flagiello A, Monti M, Vinci F, Marino G, Lilie H, Schwarz E, Rudolph R (2001). Pro-sequence assisted folding and disulfide bond formation of human nerve growth factor. J. Mol. Biol. 305: 523-533.
[192] Lee R, Kermani P, Teng KK, Hempstead BL (2001) Regulation of cell survival by secreted proneurotrophins. Science 294: 1945-1948

Other beneficial aspects could include a role in angiogenesis, in the release of growth factors sequestered by the ECM or in the subtle processing of cell-cell recognition molecules that allow repair (for review, see Yong and colleagues, 2001[180]).

In the future, all these approaches and further investigation will lead to the emergence of an integrated view on proteases in neurobiology and will provide a theoretical basis for modulating proteolytic activities, mainly, to achieve therapeutic goals in neurodegenerative diseases.

4.2 Material & Methods

4.2.1 Immunocitochemistry

Cell identity and morphology were evaluated after the immunocytochemical labelling of the cortical neurons with an anti-MAP-2 antibody, and for the astrocytes with an anti-GFAP, as described in detail in the preceding chapter in section "3.2 Material & Methods" (appendix: Protocol III). For additional cellular localization of MMP-2, neuronal cultures were incubated at a dilution of 1/200 of rabbit polyclonal anti-MMP-2 antibody (H-76, Santa Cruz Biotechnology).

4.2.2 MMPs Activities

Conditioned media of cell cultures were collected 24 hours, 4 and 7 days after culture establishment and analyzed by gelatin zymography[193] (appendix: Protocol IV).

Briefly, media samples containing 100µg of proteins were mixed with 2x SDS sample buffer [0.25M Tris (pH 6.8), 5% (w/v) SDS, 20% glycerol] and electrophoresed directly without boiling, under nonreducing conditions, on 10% polyacrylamide gels containing 0.1% gelatin.

The electrophoresed proteins were renatured at room temperature for 30 minutes in a 2.5% v/v Triton X-100 solution, before incubating at room temperature for 30 minutes in developing buffer [50mM Tris (pH 7.6), 0.02M NaCl, 5mM $CaCl_2$].

Gels were incubated in fresh developing buffer overnight at 37°C. The gels were stained (1% Coomassie Blue R-250, Sigma), distained in 25% methanol, 10% acetic acid and MMPs activity demonstrated by the presence of clear bands upon a blue background.

Quantification of gelatinolytic activities, active MMP-2 and transcripts expression was performed on at least four independent experiments.

[193] Liota, L.A. and W.G. Stetler-Stevenson (1990) Cancer Biology, 1, 96-106

4.2.3 RNA preparation and RT-PCR

A. Isolation System

Total RNA from cultures (different times and conditions of incubation) was isolated using the Trizol Reagent (Life Technologies, Cat. No.15596) according to the manufacturer's instructions by a simultaneously disrupt cells and inactivate ribonucleases method, as described in protocol V (appendix).

Each RNA sample was extracted from 3 to 5 neuronal cultures of the same isolation procedure. To inactivate RNases, all solutions used were prepared with DEPC-treated water [0.1% (v/v) and incubation for 16 hours followed by autoclaving at 120ºC, 1atm, 60 minutes] and reagents were nuclease-free. Glass labware was heat-treated at 200ºC for 16 hours and plasticwear was carefully washed with chloroform[194].

Briefly, cell lysates were homogenised in Trizol reagent and the mixture was separated in 3 phases by addition of chloroform. The aqueous phase, containing the RNA was transfer to a fresh tube and precipitated with isopropyl alcohol. The RNA pellet was dried and dissolved in 20µl of nuclease-free water. RNA samples were kept at -20ºC for short-term storage and at -80ºC for long-term storage.

B. Analysis of RNA

The concentration and purity of the RNA samples were determined by spectrophotometry. The optical density of convenient dilution of samples was measured at 260 nm and 280 nm. The A260/A280 ratio was calculated for every sample and compared to reference values of 2.0±0.15[195]. Samples which the A260/A280 ratio laid between the higher and lower values were considered free of contaminants such as proteins, phenol and salts.

[194] Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[195] Ausubel, FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Stuhl K (1996). Current protocols in molecular biology. New York: J. Wiley.

For samples that accomplished the purity criterion, the concentration was calculated with the formula: [RNA]µg/ml = A260 x dilution x 40.0[195].
Integrity of RNA was determined by agarose gel electrophoresis, after a careful wash of the electrophoresis apparatus with RNase free-water. The amount of RNA loaded per lane was 0.5 to 10 µg and electrophoresis were run at 5 V/cm until bromophenol blue has migrated two-thirds of the gel. Finally, gels were visualised and scanned under UV radiation.

C. Relative quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Expression studies of MMPs and TIMPs were done by determination of the relative amounts of mRNAs in total RNA extracts by relative quantitative RT-PCR. First strand cDNA was synthesized using the 1st Strand cDNA Synthesis Kit for RT-PCR-AMV (Roche) or TaqMan Reverse Transcription Reagents (Applied Biosystems) from 1mg of total RNA that has been confirmed to be pure and non-degraded. The reverse transcription (RT) reaction conditions were 25ºC for 10 minutes, 48ºC for 35 minutes, 95ºC for 5 minutes. Specific primer pairs were designed based on sequences deposited in databases having in mind co-amplification with β-actin fragment. Primer sequences are listed in Table 4.1. To test for differences in total RNA concentration among samples, mRNA level for rat β-actin were also determined by RT-PCR.
Thus, multiplex PCR reactions were performed in 1.25µl of cDNA as template, with 3mM $MgCl_2$, 0.2mM dNTP mix, 0.4 µM of each primer (and 36pM for β-actin).
PCR program for conditions were MMP-2, TIMP-1 and TIMP-2 was as follows:

i) Denaturation: 94ºC, 30 seconds;
ii) Annealing: 55ºC, 30 seconds;
iii) Extension: 72ºC, 1 minutes;
iv) Cycle number: 29 cycles;
v) Last extension step: 72ºC, 10 minutes.

For MMP-9, the program was similar except for annealing temperature of 57ºC and the number of cycles of 34.

Table 4.1 – Specific primer pairs sequences

Gene	Primer sequences
MMP-2	5'-GATTGACGCTGTGTATGAGG-3'
	5'-AGTCTGCGATGAGCTTAGG-3'
MMP-9	5'-TGTACCGCTATGGTTACAC-3'
	5'-TCCGCGACACCAAACTGG-3'
TIMP-1	5'-CCACCTTATACCAGCGTTATG-3'
	5'-GAACAGGGAAACACTGTGCA-3'
TIMP-2	5'-GCAGATAAAGATGTTCAAAGG-3'
	5'-CAGTCCATCCAGAGGCAC-3'
β-actin	5'-GACTACCTCATGAAGATCCT-3'
	5'-ATCTTGATCTTCATGGTGCTG-3'

PCR products were separated by 1.5-2% agarose gel electrophoresis (5 V/cm), stained with ethidium bromide and scanned under UV radiation. Densitometric analysis was performed using NIH Image 1.60 software[196]. For each sample, the ratio between the fluorescence of the target gene and the internal standard was used to overcome variability between samples such as RNA quality, RNA quantification errors, and random tube-to-tube variation in PCR and reverse transcription reactions. Results are mean values of, at least, three independent experiments. Images from representative experiments are shown.

[196] NIH image 2004 [http://rsb.info.nih.gov/nih-image/download.html].

4.2.4 Western blot analysis

Western blots were performed on cell extracts fallowing the procedures as described in Protocol VI and Protocol VII (appendix).

Cells were collected from three different cultures in each condition and analyzed in triplicates. Each culture dish was rinsed three times with Krebs solution or PBS at 4°C. The cells were collected by scraping into an extraction buffer containing 25 mM Tris-HCl, 2.5mM EDTA, 2.5mM EGTA, 1% Triton X-100, supplemented with 1mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 55µM leupeptin, pH 7.4 at 4°C.

Protein concentration was determined using a BCA Protein assay kit (Pierce) and 35 µg of extract were boiled for 5 min after adding 2x concentrated sample buffer (100mM Tris-Bicine, 2% β-mercaptoethanol, 2% sodium dodecyl sulphate, SDS). Equals amounts of protein were separate by electrophoresis (appendix, Protocol VI) on a 7.5% SDS-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes (appendix, Protocol VII).

These were then blocked for 1 hour at room temperature in 5% skim-milk (w/v) in Tris-buffered saline (137mM NaCl, 20mM Tris-HCl, pH 7.6) containing 0.1% Tween 20 (TBS-T) before immunoblotting.

Blocked membranes were incubated in TBS-T overnight at 4°C with primary antibodies (rabbit polyclonal anti-laminin 1/500, rabbit polyclonal anti-MMP-2 (H-76) 1/200, mouse monoclonal anti-MMP-9 1/500 and rabbit polyclonal anti-β-actin (H-300) 1/500). Rabbit polyclonal antibody against β-actin was used as a loading control for Western blotting.

After three washes with TBS-T, the membranes were incubated for 2 hours at ambient temperature with the HRP labelled second antibody, diluted 1:1000 in TBS-T.

The membranes were then washed three times with TBS-T and detection was carried out with ECL reagents (Amersham Pharmacia Biotech) following the manufactures instructions.

4.2.5 Mechanical lesions and primary cultures treatments

Embryonic rat neurons were precultured for 7 days to allow neurons to elaborate neuritis. Neuronal lesions were performed following the model of stylet transaction, described by Morrison and co-workers[197]. Subsequently, all culture treatments were carried out[198], by adding pan-laminin antibody (1:200) and RGD peptide (50 µM) to the culture medium.

The effects of RGD-peptide and anti-laminin antibody on neurite outgrowth were determined, 24 hours post-lesion, both by analysing the number of neurons with neurites close to the scratched vicinity and by measuring the neurite length of randomly selected neurons. Neurites of growing neurons were visualized after immunolabelling as described under immunocytochemistry.

Gelatinase activity and MMPs and TIMPs mRNA expression were also investigated.

4.2.6 Data analysis

Gelatinolytic activities on zymograms, mRNA expression and western blot analysis were expressed as a percentage of the controls ± SEM. Student's t-tests were used for statistical analysis.

[197] Morrison B, Saatman KE, Meaney DF, Mcintosh TK (1998). *In vitro* central nervous system models of mechanically induced trauma: a review. J Neurotrauma 15(11):911-928

[198] Costa S, Planchenault T, Charriere-Bertrand C, Mouchel Y, Fages C, Juliano S, Lefrancois T, Barlovatz-Meimon G, Tardy M (2002). Astroglial permissivity for neuritic outgrowth in neuron-astrocyte cocultures depends on regulation of laminin bioavailability. Glia 37(2):105-13.

4.3. Results & Discussion

Although the role of MMPs and TIMPs in the morphogenesis of non-neural tissues has been investigated, to date few studies have analyzed their expression during CNS development. Even though, as mention above these extracellular molecules are closely related with neuritogenesis and sprouting[191,192,199]. Before analysing their expression/activity patterns, some morphological studies were carried out, fallowing the procedures described in the section of Immunocytochemistry of Material & Methods of chapter 3. The immunofluorescence images shown in Fig. 4.1 reveal that neurons isolated with cardosins (or with trypsin) and cultured exhibited a normal morphology, with neurites extending from the cell bodies, evidencing a normal cellular growth, as a result of re-expression of neuritogenesis, apparently without damage. Furthermore, double labelling studies have clearly revealed MMP-2 expression by neurons in both types of culture.

4.3.1 Expression and activity of ECM proteins

Since growth development, in particular neuritogenesis, are closely related with proteolytic activity of ECM, expression/activity experiments were performed.
MMPs are expressed and secreted as inactive precursors that are activated by the removal of an N-terminal propeptide[200,201,202]. The latent and active forms of each enzyme can be differentiated on the basis of their molecular weights (MW) using zymography[198,202].

[199] Reeves TM, Prins ML, Zhu J, Povlishock JT, Phillips LL (2003). Matrix metalloproteinase inhibition alters functional and structural correlates of deafferentation-induced sprouting in the dentate gyrus. J Neurosci. 23(32): 10182-10189.
[200] Okada Y, Harris ED, Jr. & Nagase H (1988). The precursor of a metalloendopeptidase from human rheumatoid synovial fibroblasts. Purification and mechanisms of activation by endopeptidases and 4-aminophenylmercuric acetate. Biochem J 254: 731–741.
[201] Nagase H, Woessner JF Jr (1999). Matrix metalloproteinases. J. Biol. Chem. 274: 21491–21494.
[202] Vaillant C, Didier-Bazes M, Hutter A, Belin MF, Thomasset N (1999). Spatiotemporal Expression Patterns of Metalloproteinases and Their Inhibitors in the Postnatal Developing Rat Cerebellum J Neurosci. 19(12): 4994-5004.

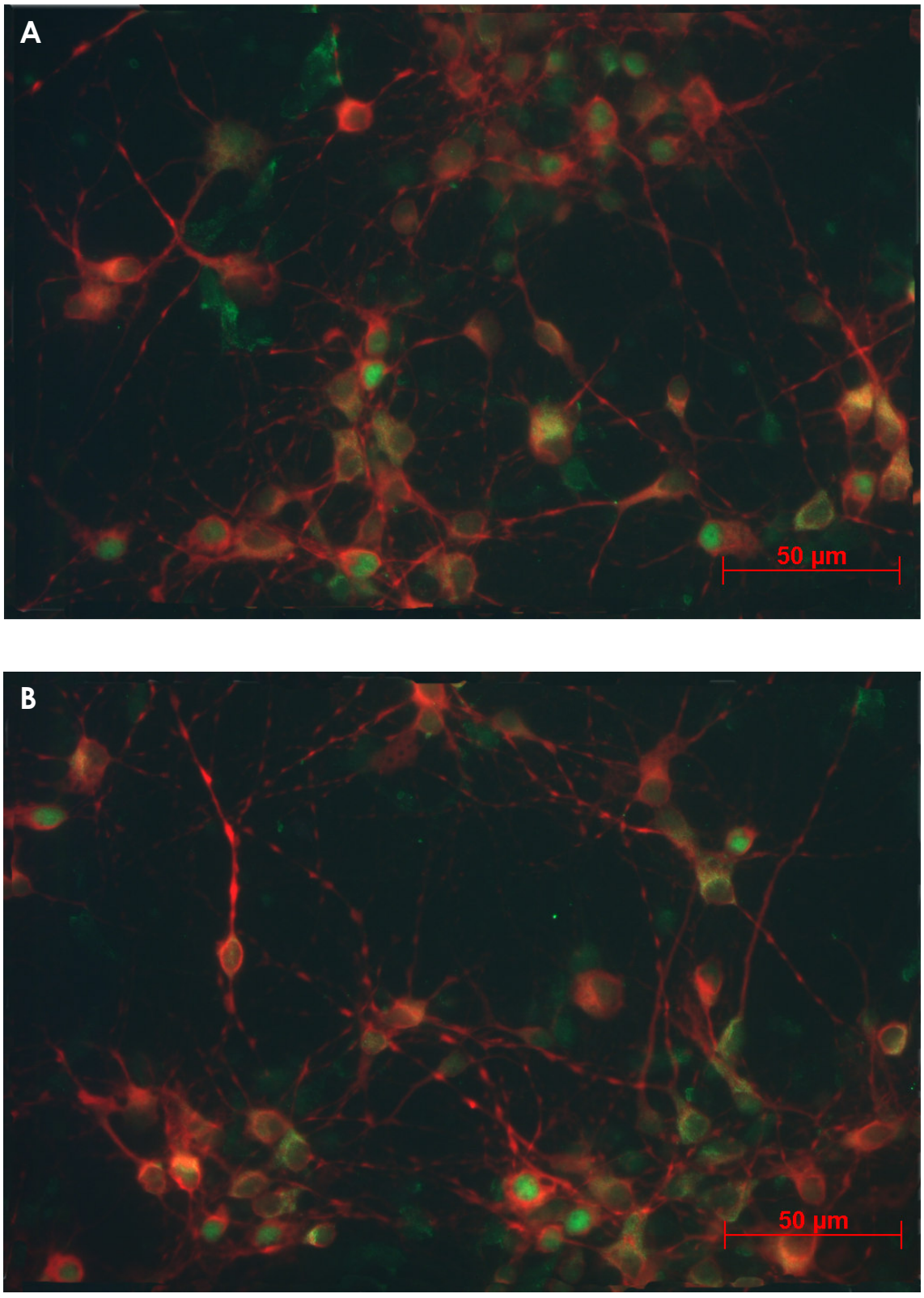


Figure 4.1 – Double immunofluorescence labelling of embryonic cortical neurons isolated with cardosins (A) or by trypsin (B). Cultured neurons for 7 days were double stained for MAP-2 (neuron marker - red) and for MMP-2 (gelatinase A - green) and consequently detected with Alexa 594-conjugated secondary anti-mouse antibody and with Alexa 488-conjugated secondary anti-rabbit antibody.

On gelatin zymography, the detect gelatinolytic activity present in culture medium corresponds to the active form of MMP-2. Results for pro-MMP-2 activity were not perceptible.

Fig 4.2 shows a zymography gel where it can be clearly notice, for the first day in culture, a great gelatinolytic activity of cultured neurons isolated with cardosins. In contrast, it can be visualised a weak band resulted from a smaller amount of active gelatinases present on conditioned medium of cultures ascertain with trypsin.

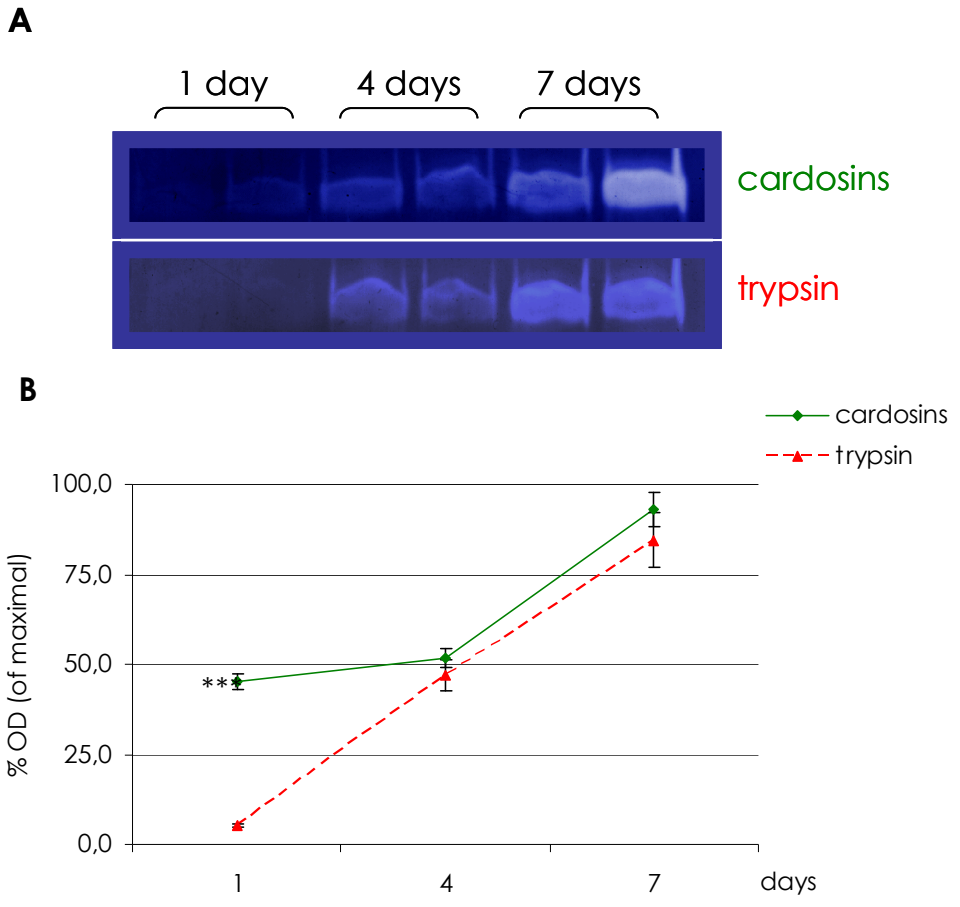


Figure 4.2 – Gelatin zymographic analysis in the conditioned media of primary neuronal cultures. A - Representative gelatin zymography of cultures ascertain by using cardosins versus trypsin (100 µg of conditioned media were analysed for the two different types of cultures at 1, 4 and 7 days after culture establishment). The proteolytic bands correspond to active-MMP-2 and the experiments show a great activity for cultures established with cardosins, in contrast to cultures generated with trypsin. B - statistical analysis of the MMP-2 densitometric data presented as a percent of maximal activity. The results (mean ± SD) shown are from 5 independent tissue samples. Statistically significant differences from controls are designated by ***, P<0.01.

On these gels, it was not detected gelatinolytic activity corresponding to the 92-kDa type IV collagenase, MMP-9, or also called, gelatinase B.

In fact, MMP-9 expression during physiological development is very low when compared to other molecules under study, but MMP-9 transcriptional overexpression is strongly induced in brain pathological states[203].

Besides regulation by zymogene activation, most MMPs are regulated at transcriptional level and their activity is also regulated by specific inhibition (TIMPs). As it has been described beforehand, several evidences indicate that the expression of MMP-9 is very low in non-pathological conditions. For that reason, for analysis of expression of these genes, a sensitive method should be selected. In view of the fact that gelatinolytic activity was not perceptible by zymography, investigation was conduced in order to examine MMP-9 expression.

Reverse transcription coupled with polymerase chain reaction (RT-PCR) is a method developed after hybridisation-based techniques have been applied to expression analysis[204], and one of its remarking features is the high sensitivity, which is provided by the amplification step. The RT-PCR technique allows several modifications that can be followed to estimate absolute or relative quantities of mRNA's. In relative quantitative RT-PCR (also termed semi-quantitative RT-PCR) an internal standard, usually cDNA from mRNA of a housekeeping gene, is co-amplified with the target cDNA in a non-competitive fashion and relative amounts of the target mRNA are obtained by comparing with the internal standard amplicon[205,206,207]. Relative quantitative RT-PCR was used to study the expression of MMP-2 and MMP-9 and their natural inhibitors, TIMP-2 and TIMP-1, respectively. Primers for reverse transcriptase reaction were specific to the poli-A tail (Oligo-dT primer) to allow the consequent quantification of mRNA.

---

[203] Lindberg RL, De Groot CJ, Montagne L, Freitag P, van der Valk P, Kappos L, Leppert D (2001). The expression profile of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in lesions and normal appearing white matter of multiple sclerosis. Brain. 124(Pt 9):1743-53.

[204] Lorkowski S, Cullen P (2003). Analysing Gene Expression: A Handbook of Methods Possibilities and Pitfalls, Vol. 2: Methods for mRNA and protein expression analysis *in situ* and *in vivo*. Weinheim : Wiley-VCH.

[205] Oliveira R, Lucas C (2004). Expression studies of GUP1 and GUP2, genes involved in glycerol active transport in *Saccharomyces cerevisiae*, using semi-quantitative RT-PCR. Curr Genet. 46(3):140-146.

[206] Masuda N, Tamaki Y, Sakita I, Ooka M, Ohnishi T, Kadota M, Aritake N, Okubo K, Monden M (2000). Clinical significance of micrometastases in axillary lymph nodes assessed by reverse transcription-polymerase chain reaction in breast cancer patients. Clin Cancer Res. 6(11):4176-4185.

[207] Al-Bader MD, Al-Sarraf HÁ (2005). Housekeeping gene expression during fetal brain development in the rat-validation by semi-quantitative RT-PCR. Brain Res Dev Brain Res. 156(1):38-45.

Before cDNA synthesis RNA quantity and purity was assessed by measurement of OD260/280 (as described in Material & Methods of this chapter) and by gel electrophoresis. In Fig. 4.3 is represented an example of RNA samples that have been selected for cDNA synthesis reactions.

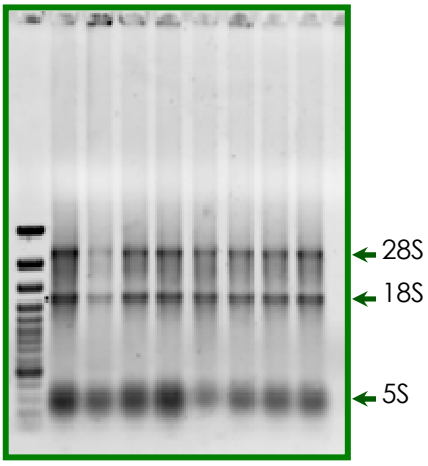


Figure 4.3 – Quality assessment of total RNA extracted from embryonic cortical neuronal cultures. The samples were run on 1.5% denaturing agarose gel. The 28S and 18S ribosomal RNA bands are clearly visible (arrows). There is no contamination with genomic DNA and it is possible to visualise a smear band that corresponds to the 5S of tRNA. Molecular weight marker used: Gene Ruler™, 100bp DNA ladder plus (Fermentas).

After cDNA synthesis, the samples were used for co-amplification of the target and the housekeeping genes. In Fig. 4.4-A is shown a representative gel electrophoresis of TIMP-2 expression performed as described under "Material & Methods" section; the multiplex RT-PCR produced a 308-base pair TIMP-2 fragment and a 490-base pair β-actin fragment with similar staining intensities. Densitometric analyses were carried out to determine the OD ratios of each lane on every performed gel. The final results are summarised on the graphical representation in Fig. 4.4-B/C. Interestly, multiplex RT-PCR results for MMP-2 and MMP-9 expression on the first day of culture are significantly different between the two isolation procedures achieving the highest levels on the neuronal cultures established with cardosins.

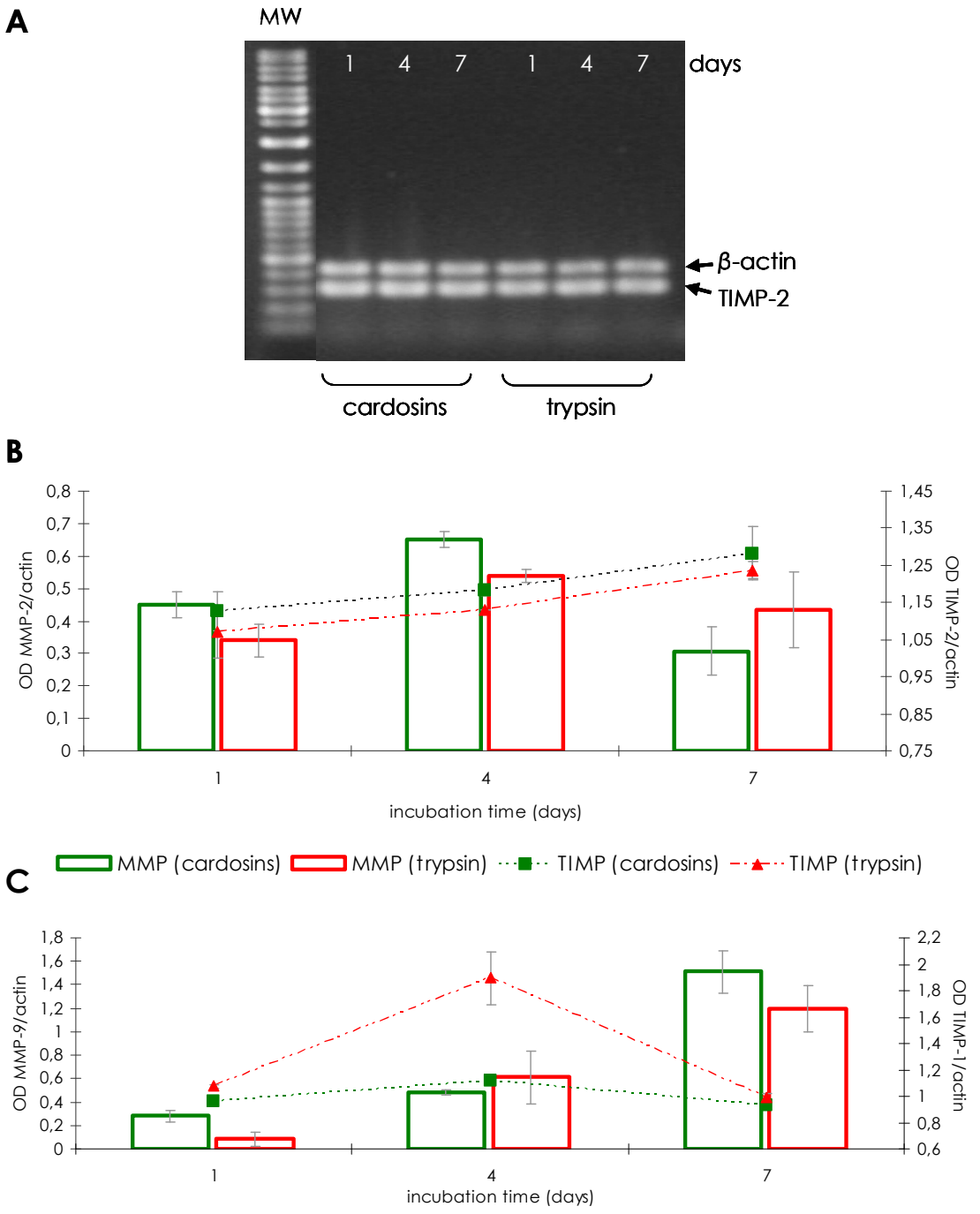


Figure 4.4 - Expression of MMPs and TIMPs on both neuronal cultures types (cardosins versus trypsin): A - representative gel electroforesis of TIMP-2 expression performed as described under "Material and Methods" section; the multiplex RT-PCR produced a 308-base pair TIMP-2 fragment and a 490-base pair β actin fragment with similar staining intensities. B) mRNA expression of MMP-2 and TIMP-2; C) mRNA expression of MMP-9 and TIMP-1. Expression values are expressed as ratio target gene/β-actin.

There are no major differences on the expression after 4 and 7 days on culture for both gelatinases.

The same is observed for TIMP-2 expression that is equally expressed during all experiments. In contrast, the homogeneity of the results of TIMP-1 mRNA expression is broken at the fourth day. As it can be observed, TIMP-1 expression by neuron isolated with trypsin is extremely high when compared to cultures established with cardosins.

Laminin is an important ECM protein that has been found to be involved in cellular activities such as neuronal migration and neurite outgrowth. To get further insights of ECM expression and since laminin is an extracellular protein that has been described as a key element on neuronal permissivity[198], besides MMPs protein level determination by western blot, laminin protein level was also assessed (Fig. 4.5). β-actin was used as an internal loading control. The results for MMP-2 and MMP-9 are consistent since the major differences are found at the first day of culture incubation. In fact, the protein expression for both gelatinases is much higher for neuron isolated with cardosins after the first 24 hours.

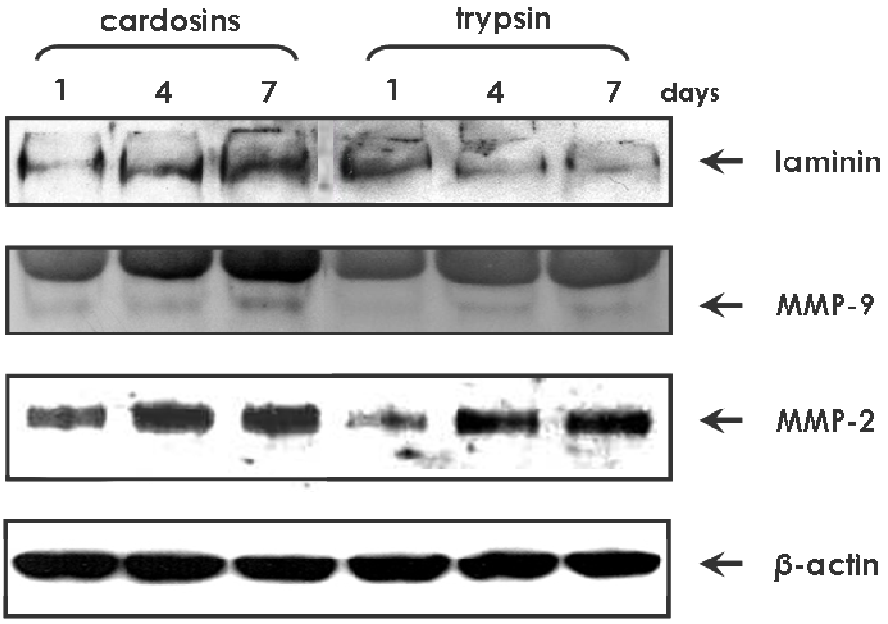


Figure 4.5 - Protein expression levels on both types of rat embryonic cell cultures (cardosins versus trypsin) assessed by western blot analysis for laminin; MMP-9: the weak band corresponds to the active form (82kDa), whereas the strong one corresponds to pro-MMP-9 (92kDa)and MMP-2: only the active form of MMP-2 was detected (60kDa). β-actin was used as a loading control.

A high level of laminin constitutes central permissive elements that can be modulated by proteolytic activity. The immunoblotting analysis detected a 200-220kDa protein band appropriate to be the size of laminin β/γ chains. Generally, during 7 days in culture neurons isolated with cardosins are expressing higher levels of laminin then neurons isolated with trypsin. Even though, after 24 hours laminin level did not vary, significantly, between the two types of culture, suggesting that this protein amount could be determinant for cell recovery from both isolation procedures. Nevertheless, immunocytochemistry analysis did not reveal significant differences of laminin distribution in both types of culture. In Fig. 4.6 it is represented an example of immunocytochemistry images obtained by double labelling studies using anti-MAP-2 and anti-laminin antibodies.

4.3.2 Anti-laminin and RGD effect on permissivity to neuritogenesis

Laminin bioavailability has been described as an important permissive factor for neuronal migration and neurite outgrowth, including postlesion situations[198,208,209,210].

In an effort to verify the ability of cell response mechanisms for both culture types, 7 days mechanically lesioned neuron-cortical cultures were treated with an anti-pan-laminin antibody to inhibit outgrowth and neuronal migration in the vicinity of a mechanical lesion.

This treatment, reported by Costa and co-workers[198] (2002), blocks substantial portion of neurite outgrowth and induces an apparent inhibition of neuronal migration toward the lesion site. This is plainly evident by analysing immunocitochemistry results where anti-laminin antibody has reduced neurite sprouting to the lesioned area.

Actually, in Fig. 4.7-c/c', immunocytochemistry analysis reveal that lesioned neuronal cultures that have been treated with anti-laminin antibody show an

[208] Rivas Rj, Burmeister Dw, Goldberg Dj (1992). Rapid effects of laminin on the growth cone. Neuron 8: 107-115.
[209] Mckeon Rj, Höke A, Silver J (1995). Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. Exp Neurol 136: 32-43.
[210] Walsh JF, Manwaring ME, Tresco PA (2005). Directional neurite outgrowth is enhanced by engineered meningeal cell-coated substrates. Tissue Eng. 11(7-8): 1085-94.

accentuate reduction of neurite outgrowth inside the lesion area when compared with untreated cultures.

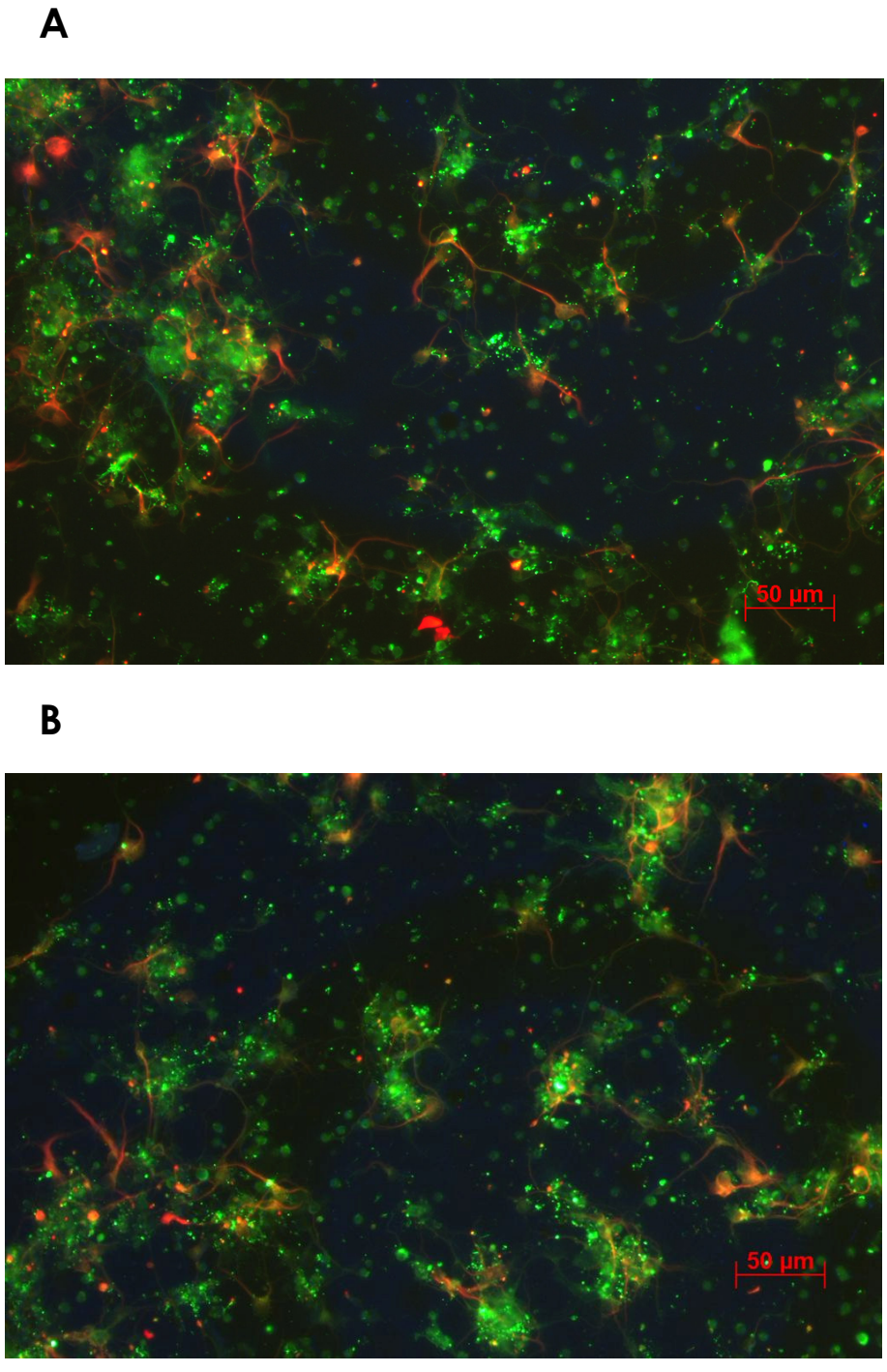


Figure 4.6 - Double immunofluorescence labelling of embryonic cortical neurons isolated with cardosins (A) or by trypsin (B). Cultured neurons for 4 days were double stained for MAP-2 (neuron marker, red) and for laminin (green) and consequently detected with Alexa 594-conjugated secondary anti-mouse antibody and with Alexa 488-conjugated secondary anti-rabbit antibody, respectively.

It has extensively been reported that ECM signalling is initiated by the interaction of distinct ECM sequences with specific cellular receptors. The best described family of ECM receptors corresponds to integrins that can interact with a number of ECM components[211]. In a few words, integrins are heterodimeric membrane proteins comprising one α and one β polypeptide chain. By using a large range of helper molecules, integrins connect the cytoplasm to the proteins on the outside of the cell[212]. But integrins are more than just adhesion molecules. They exert control over various signalling pathways to regulate virtually every aspect of cell behaviour. In cells in culture, integrins are often found in large complexes, or focal adhesions, aspects of which are thought to resemble the concentration of integrins found in juxtaposition to basement membranes. Over the past few years, evidence has emerged that integrins are crucial to brain development, yet the brain is largely devoid of classical basement membranes – it does, however, have extracellular matrix molecules[213].

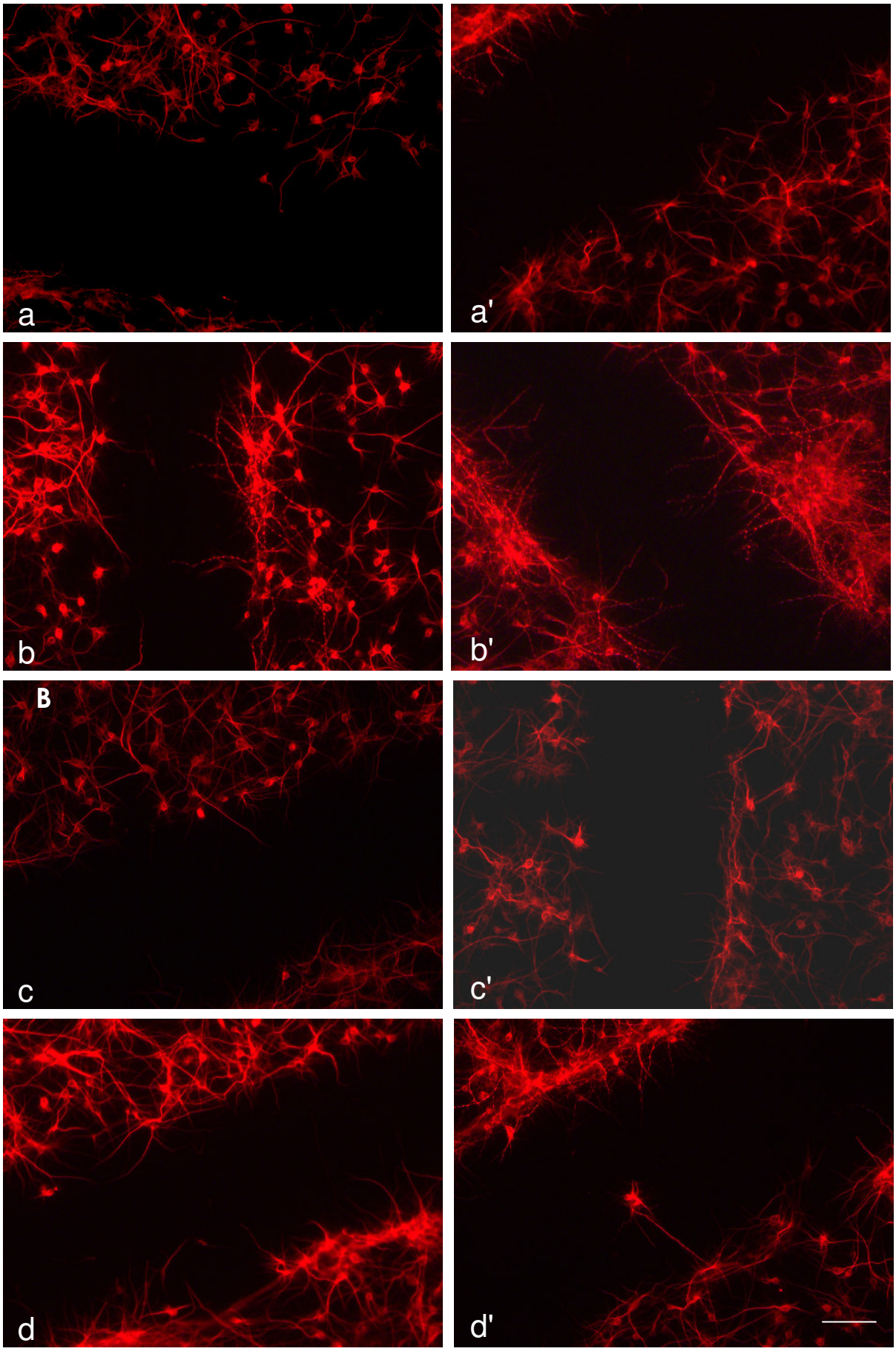
The interaction between cell and ECM components can involve different peptide sequences within the ECM molecules, among them the RGD sequence (Arg-Gly-Asp) that is contained in laminin, fibronectin, and collagen IV[214,215,216]. Consequently, peptides containing RGD sequence can be used to block cellular adhesion to RGD sequences within substrata. Similar to integrins, RGD sequences may be involved not only in adhesion but also in the promotion of neurite outgrowth[217].

Therefore, parallel assays were performed by treating mechanically lesioned neuronal cultures with RGD peptide, instead of anti-laminin antibody. Fig. 4.7-d/d' (cardosins and trypsin prepared cultures, respectively) shows that RGD effect on neuronal regrowth is closely similar to the effect observed for anti-pan-laminin antibody.

---

[211] Schwartz MA (2001). Integrin signaling revisited. Trends Cell Biol. 11(12): 466-70. Review.
[212] Van der Flier A, Sonnenberg A. Function and interactions of integrins. Cell Tissue Res 2001; 305 (3): 285-298.
[213] Novak, U. and A.H. Kaye (2000). Extracellular matrix and the brain: components and function. Review. J. Clin. Neurosci. 7, 280-290.
[214] Yamada KM (1991). Adhesive recognition sequences. J. Biol. Chem. 266: 12809–12812.
[215] Ruoslahti, E. 1996. RGD and other recognition sequences for integrins. Annu. Rev. Cell Dev. Biol. 12: 697–715.
[216] Gruenbaum LM, Carew TJ (1999). Growth factor modulation of substrate-specific morphological patterns in Aplysia bag cell neurons. Learn Mem. 6(3): 292-306.
[217] Tashiro K, Sephel GC, Greatorex D, Sasaki M, Shirashi N, Martin GR, Kleinman HK, Yamada Y (1991). The RGD containing site of the mouse laminin A chain is active for cell attachment, spreading, migration and neurite outgrowth. J Cell Physiol. Mar;146(3):451-9.

Cardosins Trypsin



(Legend in the next page)

Figure 4.7 – Blocking effect on permissivity to neuritogenesis (representative images of immunoscytochemistry). a and a') initial lesion on a 7 days culture (control); b and b') 24 h postlesion untreated culture. Observe the neurite outgrowth inside the lesion site compared with the control; c and c) 24 h postlesion treated culture with anti-laminin antibody (1/200). d and d') 24 h postlesion treated culture with RGD peptide (500µM). In c) and d) is clear the reduction of neurites projecting toward the lesion. (scale bar = 100µm). Prepared cultures by cardosins (left column) or trypsin (right column) are indicated in the figure **(Figure in the previous page).**

Gelatinase activity was also evaluated. Densitometric analysis of gelatine zymograms (data not shown), and as it can be confirmed in Fig. 4.8, have demonstrated that there is no evidence for different MMP-2 activity after 24 hours of AL or RGD treatment. Three different samples of each experimental state (treated or untreated culture cells) revealed that there is homogeneity in the results. Once again MMP-9 gelatinolytic activity was not detected.

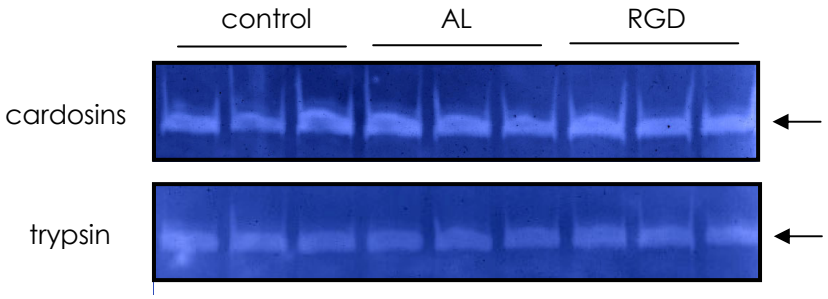


Figure 4.8- Representative gelatin zymography of either untreated (control) or treated (anti-laminin antibody, AL and RGD peptide) neuronal cultures. There are no significant differences on active form of MMP-2 (arrows) activity.

Mechanical lesioned neuronal cultures were also evaluated in terms of mRNA expression levels of ECM components that participate in axonal growth processing. It was previously mentioned that there are several evidences that MMPs and TIMPs constitute key elements on the nervous system regeneration. As well, it as been described that both TIMP-1 and TIMP-2 display growth promoting in a wide variety of cell types[218,219,220].

[218] Hayakawa T, Yamashita K, Ohuchi E, Shinagawa A. 1994. Cell growth-promoting activity of tissue inhibitor of metalloproteinase-2 (TIMP-2). J Cell Sci 107:2373–2379.
[219] Bertaux B, Hornbeck W, Eisen AZ, Dubertret L. 1991. Growth stimulation of human keratinocytes by tissue inhibitor of metalloproteinases.J Invest Dermatol 97:679–685.
[220] Stetler-Stevenson WG, Bersch N, Golde DW. 1992. Tissue inhibitor of metalloproteinase-2 (TIMP-2) has erythroid-potentiating activity. FEBS Lett 296:231–234.

Accordingly, mRNA expression of gelatinases A (MMP-2) and B (MMP-9), and TIMP-2 and TIMP-1 (their respective inhibitors) were determined by semi-quantitative RT-PCR. The results of mRNA expression 24 hours after lesion show that treatment with anti-laminin antibody led to up regulated TIMP-1 and MMP-9 expression while differences on TIMP-2 and MMP-2 mRNA expression were not detected (Fig. 4.8). The same neuronal behaviour is observed for the treatment with RGD peptide. In fact, only MMP-9 and TIMP-1 mRNA expressions are induced.

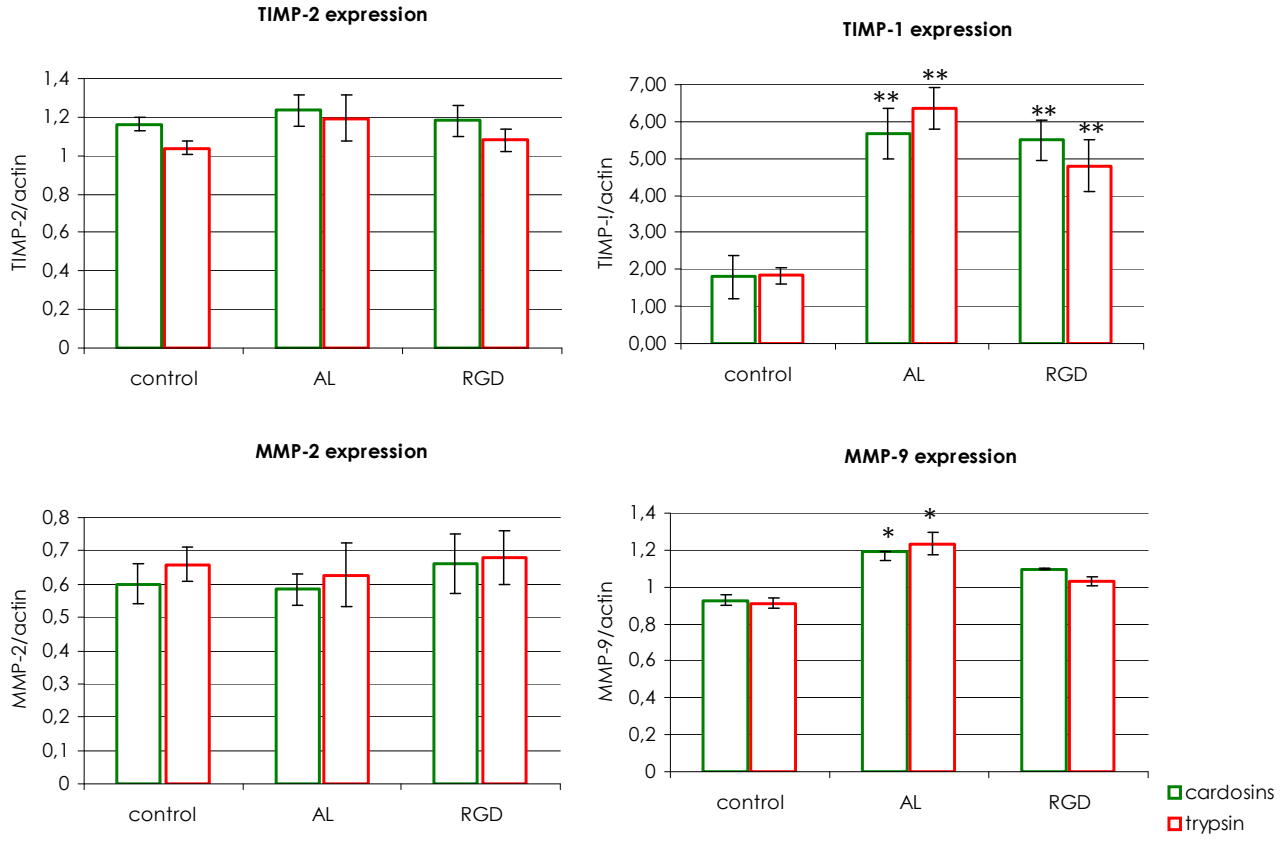


Figure 4.9 - Densitometric results from semi-quantitative RT-PCR analysis (mean ± standard deviation). Effect of anti-laminin and RGD addition to conditioned medium on mRNA expression of MMP-2, TIMP-2, MMP-9 and TIMP-1. Neuronal cultures established with cardosins (green bars) or established with trypsin (red bars) (n = 3). **P < 0.01, *P < 0.05.

At last, it is notable an increase of the extracellular laminin level for both AL and RGD treated cultures established either with cardosins or trypsin. The graphical

representation in Fig. 4.10, results from data analysis of immunodetection using anti-laminin antibody.

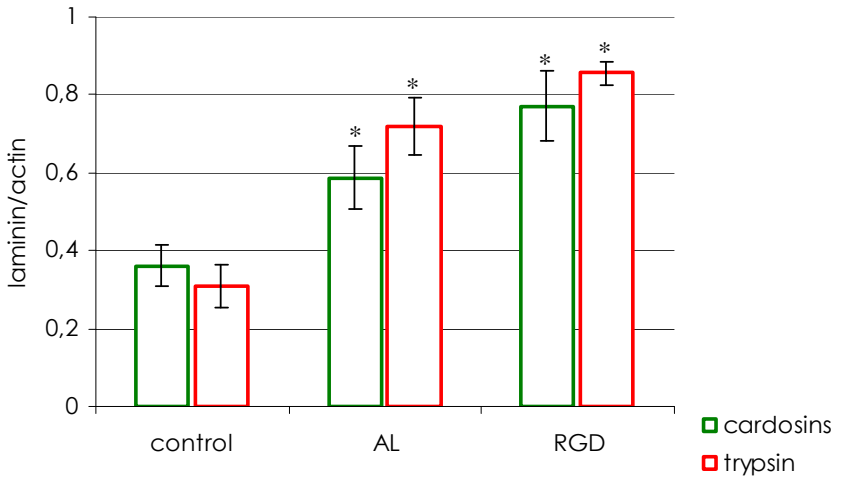


Figure 4.10 - Laminin quantification assessed by western blot analysis, (n = 3). *P < 0.05. All the described analysis were performed in parallel on both culture types (cardosins versus trypsin) showing the same results.

These results are probably associated to cell response to injury, by expressing laminin cells are producing permissive conditions that enhance neuronal growth. The present investigation sustains the idea that ECM plays a vital role in mediating axonal regeneration following brain disaggregation for establishment of primary cell cultures. Since, ECM regeneration is achieved by MMPs activity over matrix or non-matrix proteins - that direct or indirect are responsible for the rebuilt of the new network – the focal point of this investigation was to obtain the expression pattern and enzymatic activity of these molecules, after cardosins application for neuronal dissociation.

In resume, cell responses to injury, on both culture types, were comparable for mRNA expression of MMPs/TIMPs and for activity, suggesting equal cell morphology and functionality, after 7 days in culture.

Previous studies of Reeves and colleagues suggested a potentially functional role of MMP-2 in post-lesion axonal sprouting and neuronal plasticity by reporting that differentiation and subsequent functional plastic recovery is correlated with increased MMP-2 activity[199]. As well, during brain disaggregation each neuron is strained to disrupt connections to the neighbour cells resulting in a recovery-like cell response. MMP-2 expression and activity levels after 1 day of culture establishment are higher for cultures obtained with

cardosins than for trypsin cultures, suggesting that cell response to isolation procedure is faster for neurons isolates with cardosin. These results support morphological examination, previously shown in the last chapter that has demonstrated a superior number and length of neurites in cultures established with cardosins. Collectively, the results at 1 day in culture show a substantiate improvement on isolation protocol by using cardosins instead of trypsin.

Expression results for MMPs inhibitors corroborate previous findings that TIMP-2 expression increases throughout development and is maintained at high levels into adulthood[221,222,223]. Moreover, the primary function of TIMPs is to inhibit MMP activity, consequently TIMP-1 and TIMP-2 expression may be upregulated simply in response to increased MMP expression.

As far as the possible function of TIMP-1 in the brain is concerned, very little is known. However, it should be noted that this protein delivered in an adenoviral vector was capable of inhibiting excitotoxic cell death in cultured neurons[224] acting by a mechanism of neuroprotection.

The facts that TIMP-1 expression is associated with neuronal plasticity and that trypsin seems to be more aggressive to neurons during isolation procedure than cardosins, could explain the huge TIMP-1 mRNA expression obtained at the fourth day of culture for neurons obtained with trypsin.

It is well known that after injury, neurons do not spontaneously regenerate their axons, largely due to the inhibitory post-lesion environment that impedes regrowth on multiple levels. In the 1980s Aguayo and colleagues provided injured neurons in the brain and spinal cord with peripheral nerve grafts, and demonstrated that CNS neurons retain the capacity for regrowth when provided with an appropriate, permissive environment[225,226]. These guidance

[221] Fager N, Jaworski DM (2000) Differential spatial distribution and temporal regulation of tissue inhibitor of metalloproteinase mRNA expression during rat central nervous system development. Mech Dev 98: 105-109.

[222] Pérez-Martínez L, Jaworski DM (2005) Tissue Inhibitor of Metalloproteinase-2 Promotes Neuronal Differentiation by Acting as an Anti-Mitogenic Signal. J Neurosci 25(20):4917-4929)

223 Young DA, Phillips BW, Lundy C, Nuttall RK, Hogan A, Schultz GA, Leco KJ, Clark IM, Edwards DR (2002) Identification of an initiator-like element essential for the expression of the tissue inhibitor of metalloproteinases-4 (Timp-4) gene. Biochem J 364: 89-99.

[224] Tan HK, Heywood D, Ralph GS, Bienemann A, Baker AH, Uney JB (2003). Tissue inhibitor of metalloproteinase 1 inhibits excitotoxic cell death in neurons. Mol. Cell Neurosci. 22, 98–106.

[225] Aguayo AJ, David S, Bray GM. Influences of the glial environment on the elongation of axons after injury: transplantation studies in adult rodents. J Exp Biol 1981;95:231–40.

[226] Bray GM, Vidal-Sanz M, Aguayo AJ. Regeneration of axons from the central nervous system of adult rats. Prog Brain Res 1987;71:373–9.

cues are modulated by MMPs activity nevertheless further insights are needed to clarify specific roles of MMPs on permissivity.

In the present study, after 7 days in culture communication between viable cells was interrupted by mechanical lesion and re-growth of neurites and the expression of ECM proteins was measure in both types of cultures. No significant differences on the number and length of the neurites were detected. Neuronal response to CNS insults operates by selectively enhance MMP activities that improve recovery mechanisms and display neuroprotection pathways. As expected, after injury, both MMP-9 and TIMP-1 are over expressed. Although it has not been clarify whether MMP-9 is expressed by the neurons prone to death and/or those attempting to recover from the damage, it was described a potential connection(s) with neuronal survival, synaptogenesis and MMP-9[227], that supports presented data.

Following nerve injury, there is a rapid upregulation of many ECM proteins, including fibronectin[228,229] and laminin[230]. Experiments utilising function-blocking antibodies have highlighted the essential role laminin plays in promoting axonal regeneration[231,232] as it could be evaluated after morphological analysis.

MMPs are regulated after some types of insult, such as cell isolation or mechanical lesion[180]. With respect to cell migration, neural cell precursors may require balanced MMP activities in order to migrate to the scarred area. New axonal growth and synaptic reconnections need to be established and their extension through the brain matrix may also require a new MMPs/TIMPs ratio. An increase of MMP-9 and TIMP-1 expression level was evident. This new balance must contribute directly or indirectly to the increased laminin expression, which assists regrowth permissivity.

In addition to the effects on expression/activity of MMPs, it was also observed the effect of the pan-laminin antibody and the effect of RGD peptide on the morphology of cortical neurons. Surprisingly, the anti-laminin antibody

[227] Joanna Dzwonek, Marcin Rylski, Leszek Kaczmarek (2004) Matrix metalloproteinases and their endogenous inhibitorsin neuronal physiology of the adult brain. Review

[228] F. Lefcort, K. Venstrom, J.A. McDonald and L.F. Reichardt, Regulation of expression of fibronectin and its receptor, alpha 5 beta 1, during development and regeneration of peripheral nerve, Development 116 (1992), pp. 767–782.

[229] M.G. Vogelezang, S.S. Scherer, J.W. Fawcett and C. ffrench-Constant, Regulation of fibronectin alternative splicing during peripheral nerve repair, J. Neurosci. Res. 56 (1999), pp. 323–333.

[230] W. Wallquist, M. Patarroyo, S. Thams, T. Carlstedt, B. Stark, S. Cullheim and H. Hammarberg, Laminin chains in rat and human peripheral nerve: distribution and regulation during development and after axonal injury, J. Comp. Neurol. 454 (2002), pp. 284–293.

[231] E. Agius and P. Cochard, Comparison of neurite outgrowth induced by intact and injured sciatic nerves: a confocal and functional analysis, J. Neurosci. 18 (1988), pp. 328–338.

modulated neuronal morphology in a pattern very similar to the effects of RGD sequence: both treatments led to a decrease of neurite outgrowth in the scared area. Moreover, as it can be confirmed in Fig. 4.10, RGD treatment promotes laminin over-expression. One speculation arising from these observations is that both type of cultures (established with cardosins or with trypsin) act in response to the described stress conditions via laminin expression, by other words, neurons overcome injury by favouring permissivity. Finally, these data suggest that cortical neurons use RGD-dependent mechanisms for adhesion and outgrowth, witch provides possible sites of modulation by specific growth factors, since ECM components can act not only as passive substrates for neuronal attachment and outgrowth but also as active sites for signal transduction.

[232] C. Ide, Peripheral nerve regeneration, Neurosci. Res. 25 (1996), pp. 101–121.

5. MAIN CONCLUSIONS

5. Main conclusions

The results described in the present research study show that cardosins can be used to dissociate neuronal tissue to prepare cell cultures.

These aspartic proteases of plant origin allow to obtain a large number of cells, as it is achieved by using trypsin, nevertheless, this work demonstrates that cardosins use most certainly guaranties homogenous results. This is probably due to high reproducibility of cardosins purification mixture in contrast with different batches of commercial trypsin that frequently exhibit dissimilar enzymatic activities.

Cell yield and viability, and the composition in neuronal and glial cells were comparable to those obtained with trypsin digestion, but slight increase on neuronal recover after cell plating suggesting that proteolytic activity of cardosins, during tissue dissociation, follows a mechanism that is less aggressive to neurons than trypsin. This feature is, most likely, associated to a more restrict specificity of cardosins.

The results emphasise the significance of the enzymatic influence evaluation on isolation procedures, given that different enzymes can produce divergent effects on cell morphology and function by stimulating or inhibiting cell response. As different enzyme can produce different cell preparation, it seems that is important to do a pondered choice of enzymatic tool to achieve maximal cellular reliance.

Moreover, the cells isolated with cardosins are suitable for functional studies as neurotransmitter release and response to toxic injury.

The present study has demonstrated that MMPs regulation level can help to predict the cellular developing state, in terms of axonal growth and elongation, suggesting that MMPs should be viewed, not only as proteolytic enzymes acting on ECM remodelling but, overall, performing as pruning shears, playing

sophisticated roles in modulating normal cellular behaviour, cell–cell communication and tumour progression.

Actually, taking advantages of the increase on MMPs findings, these enzymes could be used as valuable tool to forecast cell behaviour and cell functional state. The broadly MMPs involvement in cell regulation mechanisms can be particularly helpful, to investigate, for instance, the effect of pharmaceutical drugs to be used in the treatment of several diseases, where MMPs might perform in an injurious manner.

As final remark, this investigation points towards cardosins application for autotransplantation procedures, as cell culture manipulation would preserve cell integrity, leading to a faster patient rehabilitation.

6. FUTURE PROSPECTS

6. Future prospects

The present work describes a combined enzymatic and mechanical method that has been optimised for rat cortex neuronal isolation for establishment of primary cell culture.

The cell yield and the physiologic condition of the neurons obtained using the isolation procedures applied during this study allows the application of these cells for diverse purposes, concerning different areas of investigation, for example, citochemistry, electrophysiology, neurotoxicology, pharmacology and therapeutics, offering greater reliance when compared to other methods, which did not include the characterization of the isolated cells were not characterised.

Clearly the use of cardosins for neuronal isolation allows obtaining cell cultures characterized by normal cellular growth.

Furthermore, this report raises the possibility of the application of cardosins for the isolation of other types of cells, taking advantage of their combined collagenolytic/gelatinolytic activity, which could be especially useful for tissues with a high content of collagen. Trypsin has been shown to be less efficient in those cases and collagenases are more expensive since they require a multi-step purification method. In contrast, the cardosins mix is obtained by a single-step purification method that yields, approximately, 10 mg of enzyme, corresponding to 5 isolation procedures. Moreover, the preparation of the cardosins can be stored as lyophilised powder. Therefore, these aspartic proteinases, by themselves or in combination with other enzymes, constitute a motivating target on cell investigation, opening new avenues for cell isolation in tissues where other enzymes were not efficient.

Finally, cardosins can be proposed as an alternative enzymatic tool for subcultivation procedures, on the establishment of finite cultures and continuous cell lines, since it has been demonstrated at this point that the use of cardosins induces less cellular damage. This feature is, most likely, associated to a more restrict specificity of cardosins, since these aspartic proteinases are characterised for hydrolysing peptide bonds between large and hydrophobic residues. Recent *in vitro* studies have shown that cardosins can hydrolyse

collagens by cleaving the molecule in some specific peptide bonds[233], which supports for cardosins behaviour during cell dissociation from tissues.

The early cell recovery to isolation procedure seems to be related to the narrow specificity of cardosins that supports their application for establishment of primary cell culture and for subcultivation procedures. In this sense, cardosins activity can be particularly interesting for auto-transplantation procedures, as cell culture manipulation would preserve cell integrity, leading to a faster patient rehabilitation.

On the topic of cardosins applications some aspects have to be considered for prospective reflection. Recombinant cardosin A should be characterized following the strategy applied during this study and the results compared with those obtained with the natural extract of cardosins. Data obtained will be essential to evaluate the commercial potential of this enzyme.

[233] Duarte AS, Pereira A, Cabrita AS, Moir A, Pires E, Barros, M (2005). The characterisation of the collagenolytic activity of cardosin A demonstrates its potential application for extracellular matrix degradative processes. Current Drug Discovery Technologies. Vol. 2, Issue 1, 37-44.

7. APPENDIX

Table 7.1 - Basal and induced MMP expression in the brain (adapted from J. Dzwonek *et al*., 2004.)

Gene	Brain structure	Expression	
		Basal level	**Upregulation (↑) / Downregulation (↓)**
MMP-2	Hippocampus	Uniformly distributed, gray matter, astrocytes	Unaffected by spatial learning Seizures (3–7 days) (↑) Sprouting (↑) Traumatic brain injury (↑) Transient ischemia (↑) Early phase of CDV infection (↑)
	Cortex	Astroglia, some neurons	Seizures (3–7 days) (↑) Transient ischemia (↑) ALS (↓)
	Cerebellum	Purkinje cells	Changes during postnatal development
	Substantia nigra		Parkinson's disease (↓)
	Pons		Spinocerebellar ataxia type 3 (↑)
MMP-3	Cortex		Transient ischemia (↑) Traumatic brain injury (↑)
	Cerebellum	Neurons, cell bodies, Bergman glia fibers	Changes during postnatal development
MMP-7	Brain	More than three orders of magnitude less than MMP-9	
MMP-8	Brain	Two orders of magnitude less than MMP-9	
MMP-9	Hippocampus	Pyramidal and granular neurons, cell bodies and dendrites	Seizures (neurons, dendrites, astrocytes) (↑) Spatial learning (↑) Sleep deprivation (↓) Alzheimer's disease (↑) Early phase of CDV infection (↑) Transient ischemia (↑)
	Cortex	Cortical neurons (cytoplasm and apical dendrites), white matter KCl	Seizures (↑) Spatial learning (↑) Depolarization (neurons) (↑) Modulated by sleep Cerebral contusion (↑) ALS (↓) Transient ischemia (↑)
	Cerebellum	Neurons, granule cell precursors, cell bodies, Bergman glial processes	Changes during postnatal development
MMP-10	Brain	Two orders of magnitude less than MMP-9	

(Cont.)

Gene	Brain structure	Expression	
		Basal level	Upregulation (↑) / Downregulation (↓)
MMP-14	Cortex		Early phase of CDV infection (↑)
	Hypothalamus		Early phase of CDV infection (↑)
MMP-15	Brain	Similar amount to MMP-9	
MMP-16	Brain	Similar amount to MMP-9	
MMP-24	Hippocampus	Pyramidal and granular neurons	Changes during postnatal development
	Cortex	Neurons	Changes during postnatal development
	Cerebellum	Neurons, cell bodies and dendrites	Changes during postnatal development

ALS, amyotrophic lateral sclerosis; CDV, canine distemper virus

Table 7.2 - Basal and induced TIMP expression in the brain (adapted from J. Dzwonek *et al.*, 2004.)

Gene	Brain structure	Expression	
		Basal level	Upregulation (↑) / Downregulation (↓)
TIMP-1	Hippocampus	Pyramidal and granular neurons, cell bodies and dendrites	Seizures (neurons, astrocytes) (↑) ECS (DG, molecular layer of DG) (↑) Early phase of CDV infection Transient ischemia (↑) Experimental autoimmune encephalitis (↑) Human T-lymphotropic virus type I infect (↑) Human immunode.ciency 1-associated dementia (↑)
	Cortex	Neurons, not astrocytes	KCl depolarization (neurons) (↑) ECS (outer layer of the cerebral cortex) (↑) Early phase of CDV infection (↑) Cerebral contusion (↑)
	Cerebellum	Neurons, Bergman glial cells	Changes during postnatal development Hypothalamus Early phase of CDV infection (↑)
	Substantia nigra		Parkinson's disease (↑)
TIMP-2			
	Cortex	Predominantly neurons	Unaffected by KCl depolarization
	Cerebellum	Neurons	Changes during postnatal development
TIMP-3	Cortex	Predominantly neurons	Unaffected by KCl depolarization Early phase of CDV infection in mousea (↑) Transient ischemia (↑)
	Cerebellum	Neurons, dendrites, cell bodies	Changes during postnatal development
TIMP-4	Cerebellum	Purkinje cells	Changes during postnatal development

CDV, Canine distemper virus; DG, dentate gyrus; ECS, electroconvulsive shock.

ECM PROTEIN SEQUENCE ANALYSIS

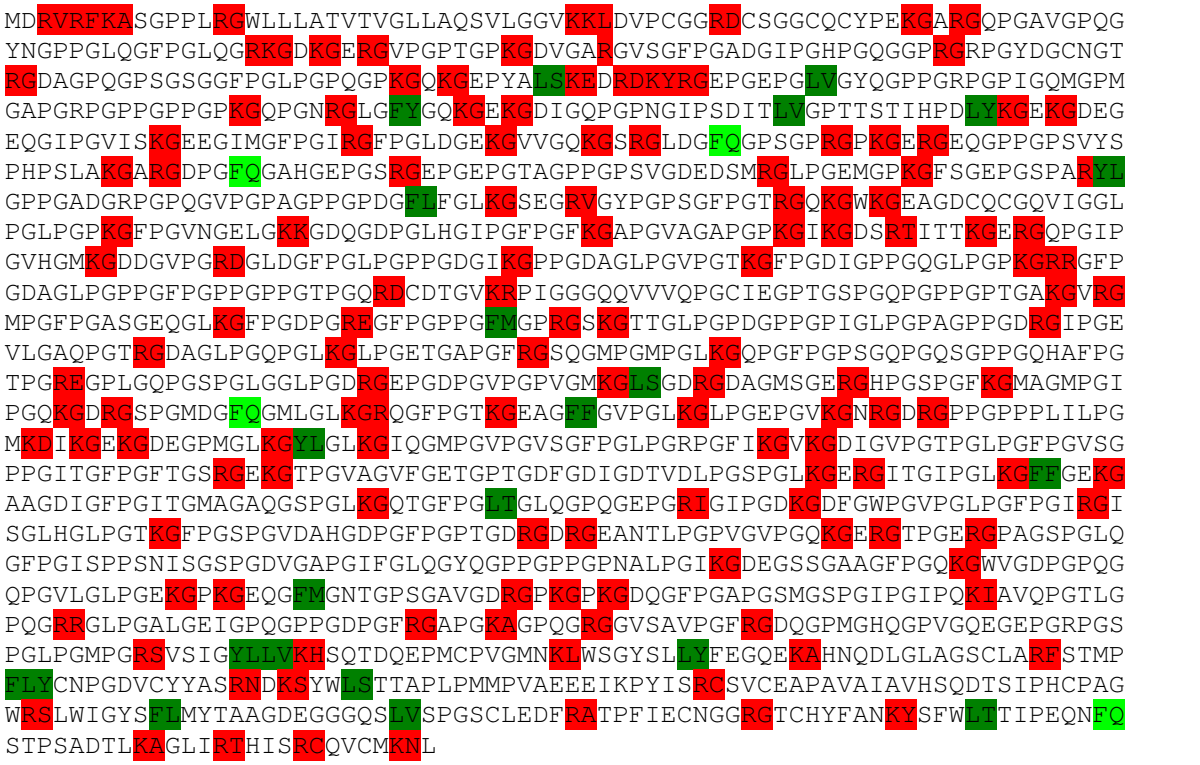


Figure 7.1 - Sequence of procollagen, type IV, alpha 2 (*Mus musculus*, NP_034062.2). Identification of hypothetical cleavage sites for cardosins (dark green) - based on their known primary specificity (F-V, L-Y, L-V, F-Y, F-M, L-T, L-S, Y-L, FF, IY) and cleavage peptide bonds for trypsin (red). Light green label peptide bond (FQ) corresponds to a cleavage site identified for type I collagen by cardosins A.

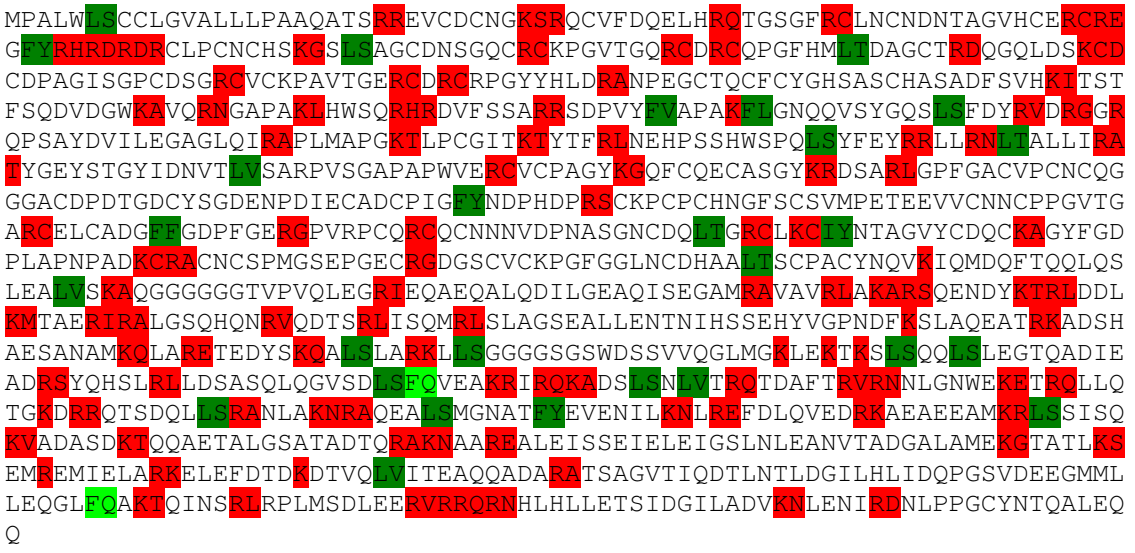


Figure 7.2 - Sequence of mouse laminin gamma-2 chain precursor, 100kDa subunit (Q61092). Peptide bond labelling as described in legend of Fig. 7.1.

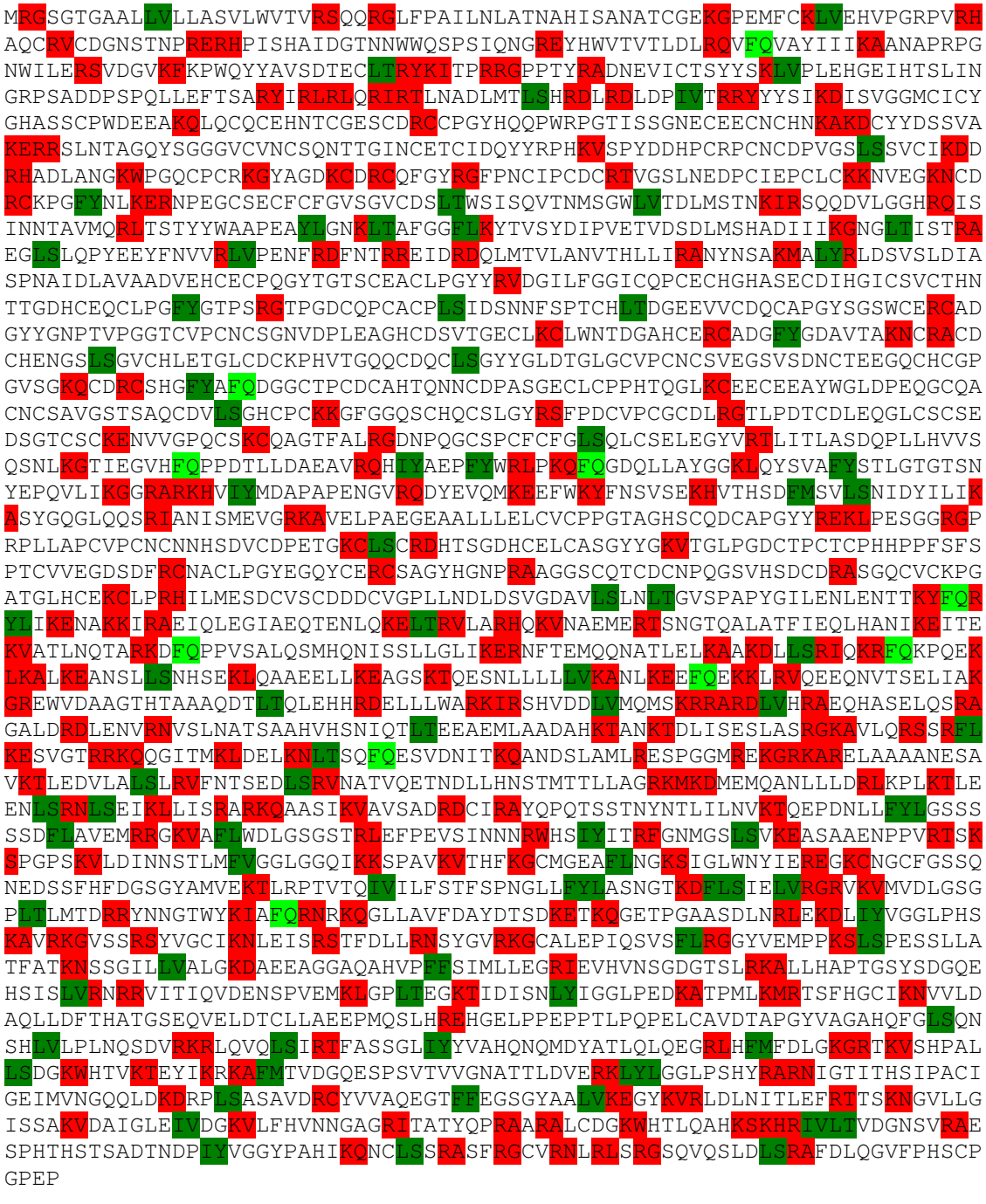


Figure 7.3 - Sequence of mouse laminin alpha-1 chain precursor, laminin A chain (P19137). Peptide bond labelling as described in legend of Fig. 7.1.

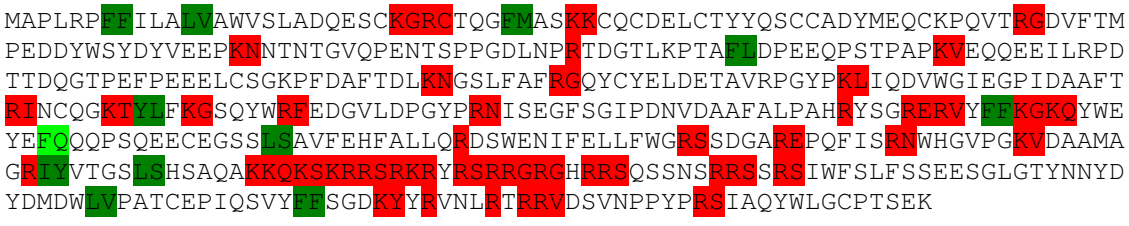


Figure 7.4 - Sequence of vitronectin (*Mus musculus*, NP_035837.1). Peptide bond labelling as described in legend of Fig. 7.1.

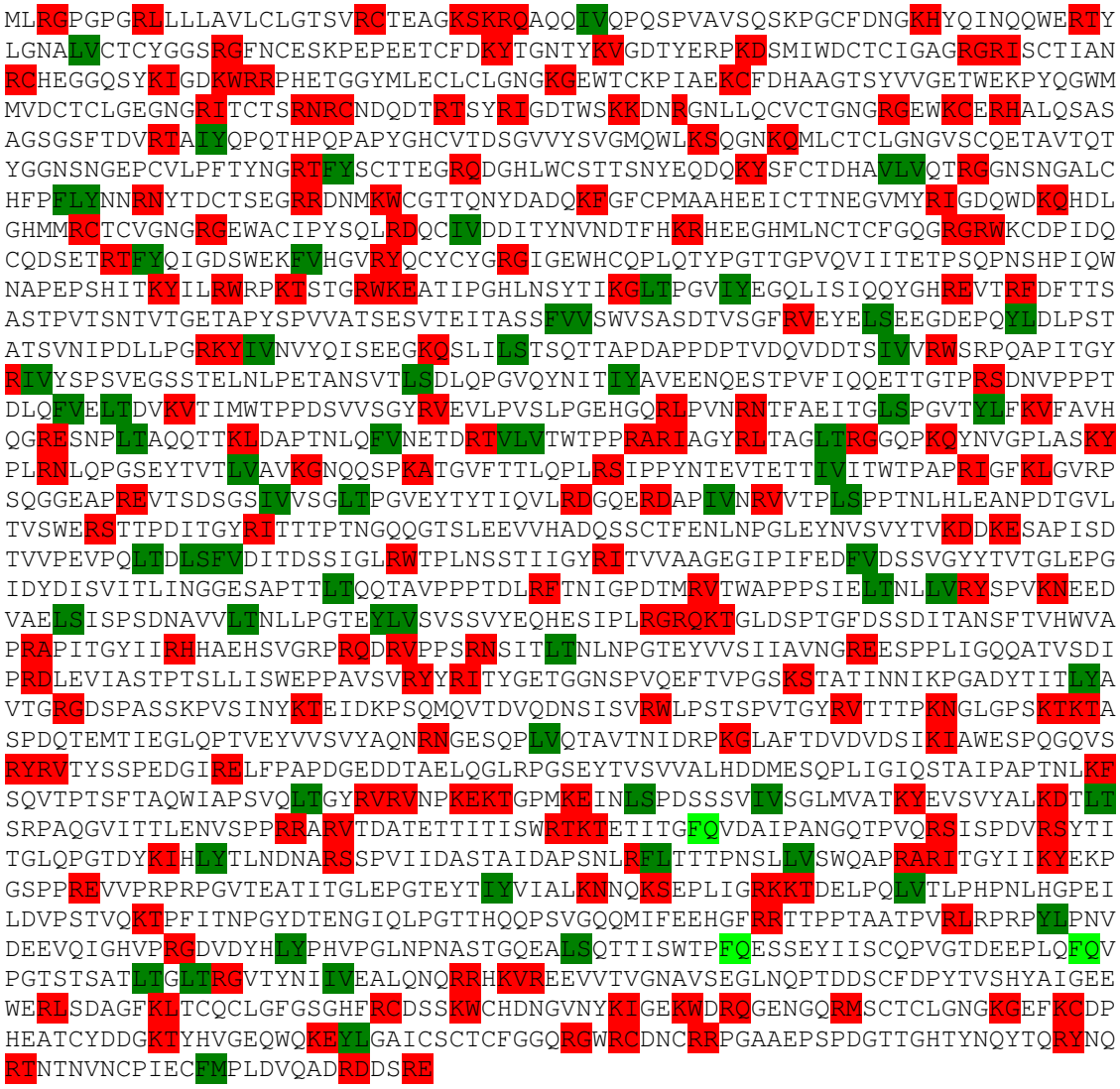


Figure 7.5 - Sequence of fibronectin 1 (*Mus musculus*, NP_034363.1). Peptide bond labelling as described in legend of Fig. 7.1.

PROTOCOL I:

PURIFICATION OF CARDOSINS

1. Cardosins Extraction from *Cynara cardunculus L.* Pistils

- Place 2g of fresh pistils in a mortar;
- Add 12ml of sodium citrate buffer, 100mM, pH 3.5;
- Ground until reduction to a homogeneous emulsion;
- Centrifuge at 14,000 rpm for 10minutes;
- Remove gently the supernatant;
- Filter the supernatant through a membrane with a 0.2µm pore. The membrane should be previously hydrated (with sodium citrate buffer, 100mM, pH 3.5). Avoid high pressure application to prevent denaturation of proteins).

2. Size Exclusion Chromatography - Hiload Superdex 75 Semi Prep

- Equilibrate the column in Tris.HCl buffer, 25mM, pH 7.6, at a flow rate of 4ml/min;
- Apply 10ml of the solution obtained in the previous step of cardosins purification to the column;
- Approximately 45min after sample injection collect the protein fraction from the column until absorbance approaches the base line; this solution corresponds to a mixture of cardosins A_0, A and B.

3. Cardosins storage

- Quantify cardosins solution;
- Prepare aliquots of 2mg, lyophilise and store at -80ºC.

PROTOCOL II:

CARDOSINS ACTIVITY

1. Hydrolysis of Lys-Pro-Ala-Glu-Phe-Phe(NO_2)-Ala-Leu synthetic peptide

- Add 21µl (63µg) of Lys-Pro-Ala-Glu-Phe-Phe(NO_2)-Ala-Leu (stock solution – 3mg/ml in reaction buffer) to 274µl of reaction buffer[234] to a final volume of 300µl.
- Incubate at 25ºC;
- Start reaction by adding 0.143µg (5µl) of cardosins;
- After 5, 10, 20, 30 minutes remove 60µl of the reaction mixture and add it to 540µl of 1.5% TFA. Mix vigorously (vortex) in order to end the reaction.

2. Analysis of hydrolysis products

- Apply the samples to a HPLC system equipped with a reverse phase column, C_{18} (250 x 4.6mm LiChroCART™ 100 RP-18, 5µm, Merck) equilibrated with 30% acetonitrile (v/v) in ultra-pure water acidified with 0.1% TFA (v/v) at a flow rate of 0.8ml/min. Detect continuously at 254nm.
- Elute the digestion products by raising acetonitrile concentration [to a final concentration of 100% acetonitrile(v/v)].

[234] Reaction buffer: 50mM Sodium acetate, 200mM Sodium chloride, 4% DMSO (v/v), pH 4.7.

PROTOCOL III:

IMMUNOCYTOCHEMISTRY

1. **Cell preparation**
   - Remove the media and rinse twice with Krebs solution or PBS.

2. **Cell fixation and permeabilization**
   - Remove the Krebs solution or PBS and incubate with 110µl of 4% PFA in PBS for 10 minutes at room temperature;
   - Rinse twice with PBS;
   - Permeabilize with 0.2% Triton X-100 in PBS for 10 minutes at room temperature;
   - Wash 4x with PBS.

3. **Gelatin-blocking**
   - Block with 0.2% gelatin in 0.5% Tween 20 in PBS for 90 minutos at room temperature;
   - Rinse once with PBS.

4. **Primary antibody incubation**
   - Add 80µl of primary antibody dilution and incubate for 90 minutes at room temperature.
   - Remove and wash 6x with 0.5% Tween 20 in PBS.

Protect from light

5. **Secondary antibody incubation**
   - Add 80µl of primary antibody dilution and incubate for 90 minutes at room temperature.
   - Remove and wash 6x with 0.5% Tween 20 in PBS.

6. **Mounting**
   - Identify and clean the glass slides with ethanol.

- Wash thoroughly the coverslips
- Pick up the coverslips with forceps and drain away the excess of buffer
- Put a drop of fluorescent mounting medium (DAKO) and gently lay coverslip on top
- Let dry for 2-3 hours at room temperature
- Seal the coverslip border with nail malt
- Store in -20°C freezer

PROTOCOL IV:
GELATIN ZYMOGRAPHY (adapted protocol[235])

1. Gel preparation

Prepare gels (7.5%, 10% or 12%) according to the standard procedure. During the running gel preparation add gelatin stock solution (10 mg/ml in H^2O) to get the gelatin concentration of 0.1% (1 mg/ml).

2. Samples preparation

Mix one part sample with one part sample buffer (2x)[(i)] and let stand 10 minutes at room temperature. DO NOT HEAT.

3. Running conditions

Apply samples (typically 10-25 µl) and run the gel with 1x Tris-Bicine SDS Running Buffer[(ii)] according to the standard running conditions (~125V, constant voltage). Run time (60-120 min) will depend on the gel percentage and running buffer concentration and pH.

4. Gel renaturation

After running, dilute the zymogram Renaturing Buffer (10x)[(iii)] 1:9 with deionized water and incubate the gel in the buffer (100 ml for one or two mini-gels) with gentle agitation for 30 minutes at room temperature.

5. Zymogram developing phase

Decant the Zymogram Renaturing Buffer and replace with 1x Zymogram Developing Buffer[(iv)] (100 ml for one or two mini-gels). Equilibrate the gel for 30 minutes at room temperature with gentle agitation then replace with fresh 1x Zymogram Developing Buffer and incubate at 37°C for at least four hours. Incubate overnight for maximum sensitivity. Incubation time can be reduced to as little as one hour for concentrated samples. The optimal result can be determined empirically by varying the sample load or incubation time.

6. Zymogram revelation

Stain with Coomassie Blue R-250 for 30 minutes. For maximum contrast, use a stain concentration of 0.5% (w/v) instead of the usual concentration of 0.1%. Gels should be destained with an appropriate Coomassie R-250 destaining solution (Methanol : Acetic acid : Water; 50 : 10 : 40). Areas of protease activity will appear as clear bands against a dark blue background where the protease has digested the substrate.

STOCK SOLUTIONS

(i) Sample Buffer	**(2X)**
0.5 M Tris-HCl, pH 6.8	2.5 ml
Glycerol	2.0 ml
10% (w/v) SDS	4.0 ml
0.1% Bromophenol Blue	0.5 ml
Distilled water to 10.0 ml	

(ii) Running Buffer (10x)	**(1X)**
Tris Base 29 g	2.9 g
Glycine 144 g	14.4 g
SDS 10 g	1.0 g
0.1% Bromophenol Blue	0.5 ml
Distilled water to 1000 ml	pH=8.3

(iii) Renaturing Buffer	**(10x)**
Triton X-100, 25% (v/v) in water	2.9 g

(iv) Developing Buffer (10x)	**(1X)**
Tris base, 12.1 g Tris-HCl, 63.0 g	50mM
NaCl, 117 g	0.2M
CaCl2, 7.4 g	5 mM
Distilled water to 1000 ml	

[235] Liotta, L.A. and W.G. Stetler-Stevenson. Metalloproteinases and cancer invasion. (Review -1990). Semin. Cancer Biol.1(2): 99-106.

PROTOCOL V:

RNA ISOLATION USING TRIZOL PROCEDURE

1. Homogenization

Lyse cells grown in monolayer directly in a culture dish by adding 1 ml of TRIZOL Reagent to a 3.5 cm diameter dish (mix gently by passing the cell lysate several times through a pipette).

2. Phase separation

Incubate the homogenized samples for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform per 1 ml of TRIZOL Reagent. Cap sample tubes securely. Shake tubes vigorously by hand for 15 seconds and incubate them at room temperature for 2 to 3 minutes. Centrifuge the samples at no more than 12,000 x g for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIZOL Reagent used for homogenization.

3. RNA precipitation

Transfer the aqueous phase to a fresh tube[236], and precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization. Incubate samples at room temperature for 10 minutes and centrifuge at no more than 12,000 x g for 10 minutes at 2 to 8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

4. RNA wash

Remove the supernate. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization.

Mix the sample by vortexing and centrifuge at no more than 7,500 x g for 5 minutes at 2 to 8°C.

[236] save the organic phase if isolation of DNA or protein is desired.

5. Redissolving the RNA

At the end of the procedure, briefly dry the RNA pellet (air-dry or vacuum-dry for 5-10 minutes). Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Dissolve RNA in RNase-free water by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55 to 60°C.

NOTES:

Reagents required:

- Chloroform
- Isopropyl alcohol
- 75% Ethanol (in DEPC-treated water)
- RNase-free water or 0.5% SDS solution [To prepare RNase-free water, draw water into RNase-free glass bottles. Add diethylpyrocarbonate (DEPC) to 0.01% (v/v). Let stand overnight and autoclave. The SDS solution must be prepared using DEPC-treated, autoclaved water.]

Precautions for Preventing RNase Contamination:

- RNases can be introduced accidentally into the RNA preparation at any point in the isolation procedure through improper technique. Because RNase activity is difficult to inhibit, it is essential to prevent its introduction. The following guidelines should be observed when working with RNA.
- Always wear disposable gloves. Skin often contains bacteria and molds that can contaminate an RNA preparation and be a source of RNases. Practice good microbiological technique to prevent microbial contamination.
- Use sterile, disposable plasticware and automatic pipettes reserved for RNA work to.

PROTOCOL VI:

ELECTROPHORETIC SEPARATION OF PROTEINS IN DENATURING CONDITIONS (SDS-PAGE)

1. Sample preparation

- Place the multiwell plate on ice to proceed with cell lysis (for protein extraction).
- Remove the media and rinse twice with Krebs solution or PBS.
- Thoroughly, scraping the cells off the plates and resuspending them in lysis buffer[237].
- Dilute samples with 35-40µg of total protein in denaturing solution[238] (1:1; v:v), heat them at 100ºC for 3-5min and apply to the polymerised gel. Use an appropriate molecular weight pre-stained marker.

2. Running gel – 7.5%

Add the following chemicals sequentially:

- 4.765ml mili-Q water;
- 3.36ml Tris 1.5M pH 8.8;
- 1.875ml acrylamide:bisacrylamide (40%, w/v);
- 200µl SDS (10%, w/v);
- 100µl ammonium persulphate (10%, w/v);
- gently homogenise;
- 5µl TEMED.

Apply the homogenised solution, immediately, to the assembled system, place the combs on the top of the gel and allow it to polymerise.

3. Running buffer

Apply samples (35-40 µg) and run the gel with 1x Tris-Bicine SDS Running Buffer[239] according to the standard running conditions (~125V, constant voltage). Run time (60-120 min) will depend on the molecular weight of the protein under study.

[237] Lysis buffer: 25mM Tris, 2.5 mM EDTA, 2.5 EGTA, 1% Triton X-100, supplied with 1mM DTT, 1mM PMSF and 52.57µM leupeptin.
[238] Denaturing solution: 2% β-mercaptoethanol (v/v); 2% SDS (w/v); 8M Urea; 100mM Tris:Bicine
[239] SDS-Runing buffer: 100mM Tris:Bicine; 0.1% SDS (w/v).

PROTOCOL VII:

WESTERN BLOT- IMMUNOBLOTTING PROPOSES
(Wear gloves to avoid contamination)

1. **Assembling Western blotting apparatus**
   - Cut a piece of nitrocellulose membrane and two pieces of filter paper (3MM) to the dimensions of the gel;
   - Equilibrate the gel and soak the membrane, filter paper (3MM) and fiber pads in transfer buffer[240] (15min);
   - Prepare the gel sandwich as follows (Figure 7.1):

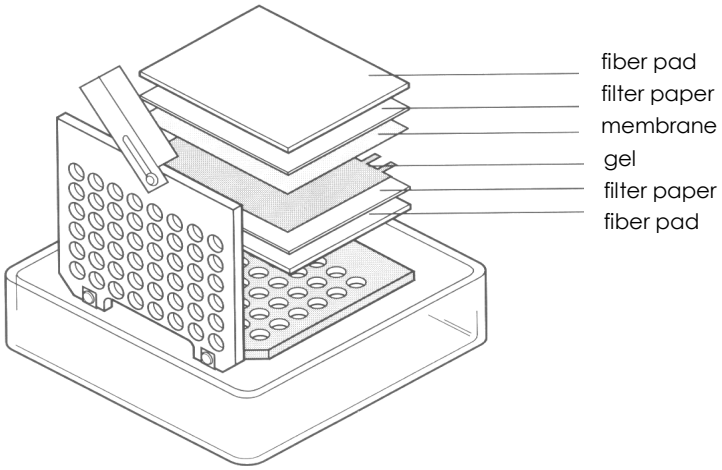


Figure 7.6 - Schematic representation of how to assemble a blot sandwich (Bio-Rad, instructions manual): a) Place one pre-wetted fibber pad on the black plate of the cassette; b) Place a sheet of 3MM paper on the fibber pad; c) Place the equilibrated gel on the filter paper; d) Place the pre-wetted membrane on the gel (remove any air bubbles which may have formed; use a glass tube to gently roll air bubbles out); e) Complete the sandwich by placing a piece of filter paper on the membrane and the last fibber pad.

   - Close the cassette firmly, being careful not to move the gel and filter paper sandwich.
   - Place in tank and completely fill the tank with buffer (attention: black plate of the cassette must be placed next to negative pole).
   - Add a stir bar (to help maintain even buffer temperature and ion distribution in the tank) and run the blot.

[240] Transfer buffer: 25mM Tris, 192mM Glicine, pH 8.2; 10% (v/v) methanol.

2. Transfer conditions

- 40mA (constant);
- ~17 hours;

The transfer tank was coupled to a refrigerated unit to improve heat dissipation. These conditions are suitable for high molecular mass proteins.

3. Disassemble transfer apparatus

- Carefully disassemble the transfer apparatus. Using tweezers or forceps, place the nitrocellulose membrane in container and wash for 10 min in 0.1% TBS-T[241].

4. Blocking membrane

- Block the membrane with 5% skim milk in 0.1% TBS-T for 1-2 hours.

5. Primary antibody incubation

- Prepare the primary antibody using the recommended dilution in 0.1% TBS-T, 1.5% skim milk;
- Incubate overnight with gentle rocking in cold room.

6. Secondary antibody incubation

- Wash three times for 5 minutes with 0.1% TBS-T;
- Dilute the secondary antibody to the desired concentration in 0.1% TBS-T and incubate for 1-2 hours with gentle rocking at room temperature;
- Wash the membrane three times in 0.1% TBS-T.

Protect from light

7. Visualise with ECL

- Prepare ECL solution for detection: mix equal volume of ECL reagent 1 and 2, (1:1) with final volume regarding of 0.125 ml/cm^2;
- Remove excess buffer from the membrane by draining the membrane over a piece of filter paper and briefly touching the edge of the membrane to

[241] Tris-buffered saline with 0.1% Tween 20

the paper. Add the mixed ECL reagent to the surface of the membrane for 1 minute;

- Drain off the ECL reagent and wrap the blot in a plastic (transparent) wrap;
- Tape membrane to the inside film cassette;
- Add 1 sheet of x-ray film (Kodak) to the cassette. Expose membrane to film. It will probably be necessary to do several different exposures to find out the best exposure.
- Develop the film.