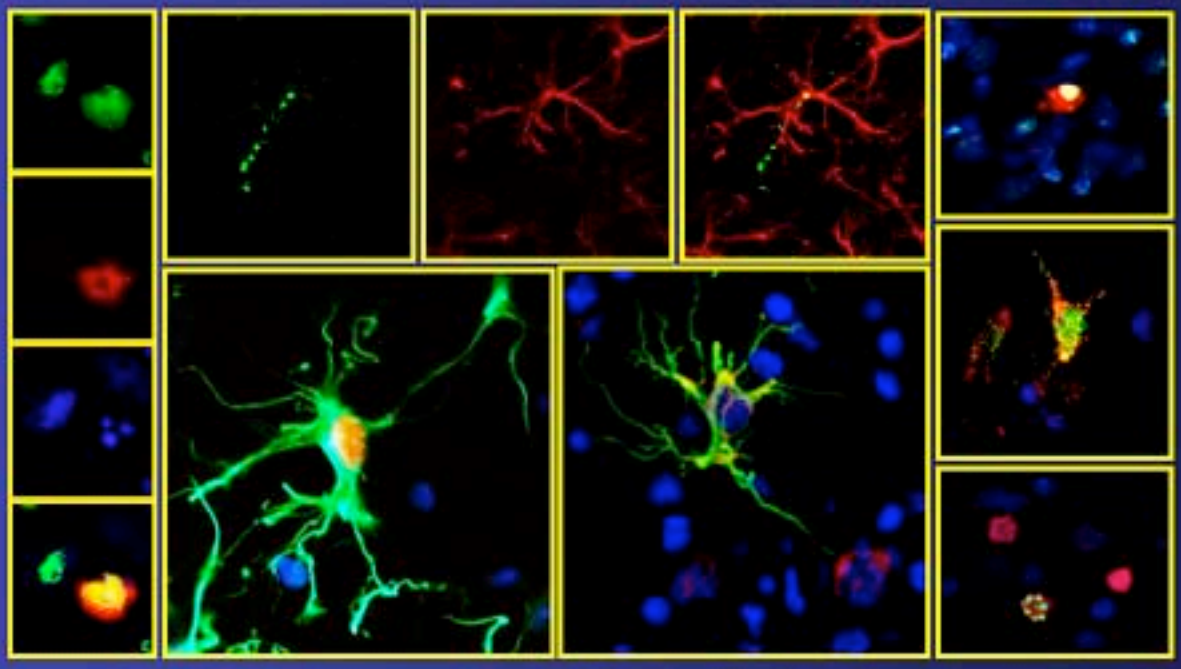
Universitat Autònoma de Barcelona

Institut de Neurociències

Apoptotic and Antiapoptotic mechanisms in neurons and glial cells after damage to the immature brain



DOCTORAL THESIS

Sonia Villapol Salgado

September 2007

Universitat Autònoma de Barcelona

Departament Cell Biology, Physiology and Immunology.
Institute of Neurociences.
Faculty of Medicine. Autonomous University of Barcelona. Bellaterra, Barcelona. Spain.

APOPTOTIC AND ANTIAPOPTOTIC MECHANISMS IN NEURONS AND GLIAL CELLS AFTER DAMAGE TO THE IMMATURE BRAIN

Doctoral Thesis

September 2007

Sonia Villapol Salgado

www.svstjt.com

Cover photograph:
Fluorescence pictures collage; caspase-9, caspase-8, astrocytes with caspase-3, astrocytes with survivin, astrocytes with chaperones, astrocytes with cleaved filaments, and neurons dying.

Printed in Spain by UAB
Barcelona 2007

Universitat Autònoma de Barcelona

Facultat de Medicina

Medical Histology
Dept. Cell Biology, Physiology and Immunology
Institute of Neurosciences
Autonomous University of Barcelona

LAIA ACARIN PEREZ i BERTA GONZÁLEZ, Professoras titulars del Departament de Biologia Cel·lular, Fisiologia i Immunologia de la Universitat Autònoma de Barcelona.

CERTIFIQUEN: que la tesis "APOPTOTIC AND ANTIAPOPTOTIC MECHANISMS IN NEURONS AND GLIAL CELLS AFTER DAMAGE TO THE IMMATURE BRAIN", que presenta Sonia Villapol Salgado per optar al grau de Doctora ha estat realitzada sota la seva direcció al Departament de Biologia Celular, Fisiologia i Immunologia de la Universitat Autònoma de Barcelona i es troba en condicions de ser llegida.

Bellaterra, Setembre de 2007

Dra. Laia Acarin

Dra. Berta González

a Toddiño,

a meus pais,

a Ito.

... la ansia por investigar es arrancarle secretos a la vida, ... el descubrimiento científico, el encontrar algo que uno de momento se da cuenta que es el primero que lo ve y que es algo con una trascendencia ... yo no conozco nada que produzca más emoción.

Severo Ochoa (1905-1993)

Dans la vie, rien n'est à craindre, tout est à comprendre.

Marie Curie (1867-1934)

TABLE OF CONTENTS

I. LIST OF ARTICLES

This thesis is based on the following articles:

ARTICLE I Acarin L., Villapol S., Faiz M., Rohn T.T., Castellano B. and Gonzalez B. "*Caspase-3 activation in astrocytes following postnatal excitotoxic damage correlates with cytoskeletal remodeling but not with cell death or proliferation*". GLIA 55:954 - 965 (2007).

ARTICLE II Villapol S., Acarin L., Faiz M., Castellano B. and Gonzalez B. "*Distinct spatial and temporal activation profile of apoptotic pathways in neurons and glial cells after excitotoxic damage in the neonatal rat brain*". J. Neuroscience Research. *In press* (2007).

ARTICLE III Villapol S., Acarin L., Faiz M., Castellano B. and Gonzalez B. "*Survivin and Heat Shock Protein 25/27 colocalize with cleaved caspase-3 in surviving reactive astrocytes following excitotoxicity to the immature brain*". *Submitted* (2007).

II. ABBREVIATIONS

AIF	Apoptosis-inducing factor
Apaf-1	Apoptotic protease activating factor
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
Bcl-*2*	B-cell lymphoma-2
BDNF	Brain-derived neurotrophic factor
BrdU	5-bromo-2′-deoxyuridine
Ca^{2+}	Calcium
CA	Cornu ammonis
CAD	Caspase-activated DNase
CARD	Caspase recruitment domain
Caspases	Cysteinyl aspartate-specific proteases
Cdc	Cell division cycle
CDK	Cyclin-dependent kinase
CNS	Central Nervous System
Cyt c	Cytochrome c
DAB	Diamino benzidine
DAPI	4′,6-diamidino-2-phenylindole
DD	Death domain
DED	Death effector domain
DEVD	Asp-Glu-Val-Asp, caspase-3 inhibitor
DG	Dentate Gyrus
DISC	Death inducing signaling complex
DNA	Deoxyribonucleic acid
$\Delta\Psi$	Mitochondrial membrane potential
FAK	Focal adhesion kinase
FADD	Fas-associated death domain
EAAT1	Excitatory amino acid transporter 1, or GLAST, glutamate transporter
EAAT2	Excitatory amino acid transporter 2, or GLT1, glutamate transporter
ECM	Extracellular matrix
EGF	Epidermal growth factor
ER	Endoplasmatic reticulum
FBS	Fetal bovine serum
GFAP	Glial fibrillary acidic protein
GIF	Growth inhibitory factor
Glu	Glutamate
mGluR	Metabotropic glutamate receptor
iGluR	Ionotropic glutamate receptor
GS	Glutamine synthetase
H_2O_2	Hydrogen peroxide
IAP	Inhibitor of apoptosis protein
cIAP-*2*	cellular inhibitor of apoptosis protein-2
ICAD	Inhibitor of CAD
IFs	Intermediate filament proteins
IL-1	Interleukin-1

IL-6	Interleukin-6
JNK	Jun amino-terminal kinase
HSP	Heat Shock or Stress Protein
MAP-2	Microtubule-associated protein-2
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
MHC	Major histocompatibility complex
NeuN	Neuronal nuclear antigen
NF-κB	Nuclear Factor-kappaB
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NO	Nitric oxide
NOS	Nitric oxide synthase
p38MAPK	p38 mitogen-activated protein kinase
PCD	Programmed Cell Death
PCNA	Proliferating Cell Nuclear Antigen
PI3K	phosphoinositide 3-kinase
PARP-*1*	Poly (ADP-ribose) polymerase-1
PLA2	Phospholipase A_2
ROS	Reactive oxygen radicals
SOD	Superoxide dismutase
STAT3	Signal Transducer and Activator of Transcription 3
SVZ	Subventricular zone
TGF-β	Transfroming growth factor-β
TNFα	Tumor Necrosis Factor- α
TBS	Tris Buffered Saline
TNFR	Tumor Necrosis Factor Receptor
TRADD	TNF-receptor death domain
TUNEL	Terminal deoxynucleotide Transferase (TdT) mediated dUTP nick end labeling
XIAP	X chromosome-linked Inhibitor of Apoptosis Protein

III. ABSTRACTS

ABSTRACT

English

Although it is well established that apoptotic mechanisms play a central role in neuronal death after different types of CNS damage, little is known about the activation of apoptotic pathways in glial cells which have been postulated to be important for the termination of the glial response. It is remarkable that the classically defined main executioner of programmed cell death, caspase-3, has recently been attributed a non-apoptotic role, participating in cellular events including cell proliferation, cell cycle regulation, or cellular differentiation. Accordingly, the general aim of this thesis was to analyze the putative role of the classic apoptotic and anti-apoptotic pathways after acute excitotoxic damage to the immature rat brain. For this purpose, a well-characterized in vivo model of excitotoxicity was used, where damage was induced by the intracortical injection of N-methyl-D-asparate (NMDA) in postnatal day 9 rats. Cell death, apoptotic and anti-apoptotic proteins were analyzed at different survival times after the lesion by enzymatic assays and double immunofluorescence for confocal microscope analysis.

The first set of results demonstrated that although cleaved caspase-3 was found in some damaged neurons showing TUNEL+ nuclei, cleaved caspase-3 was mainly observed in the nucleus of activated astrocytes in the lesioned hemisphere as early as 4 h post-lesion, persisted until the glial scar was formed at 7-14 days, and was marginally associated with TUNEL labeling. Caspase-3 enzymatic activity was significant during the first hours post-lesion and co-localized with the presence of caspase-cleaved fragments of glial fibrillary acidic protein (CCP-GFAP) in astrocytes. However, at longer survival times, when astroglial hypertrophy was observed, astroglial caspase-3 did not generally correlate with GFAP cleavage but instead was associated with de novo expression of vimentin. Moreover, astroglial caspase-3 cleavage was not associated with cell proliferation. These first results provided evidence for a non-traditional role of caspases in astroglial function, suggesting that caspase activation may be important for astroglial cytoskeleton remodeling following injury.

Secondly, in order to evaluate upstream pathways activating caspase-3 in neurons and glial cells, the spatio-temporal activation of the intrinsic and extrinsic apoptosis pathways were analyzed. In damaged neuronal cells of the ipsilateral cortex and hippocampus an important contribution of the intrinsic mitochondrial pathway through caspase-9 activation was shown to account for most of neuronal caspase-3 activation and apoptotic nuclei. However, neuronal caspase-8 showed a diminished correlation with caspase-3 and characteristically showed cortical layer specificity. In glial cells, activation of caspase-9, caspase-8, and also p53, another putative caspase-3 activating molecule, was only significant in cortical layer VI and the corpus callosum, suggesting that astroglial caspase-3 does not generally correlate with classical upstream activating pathways.

In the third part of this thesis, inhibitor of apoptosis proteins (IAPs) and heat shock proteins (HSPs), known inhibitors of cleaved caspase-3 in other cell types, were analyzed. Whereas neuronal cells showed noticeable expression of HSC70/HSP70 and cIAP-2, these poorly correlated with caspase-3 in this cell type. In astrocytes, survivin and the small HSP25/27 showed a strong correlation with caspase-3 in several regions and throughout different survival times, pointing to these proteins as relevant candidates for the blockade of caspase-3 enzymatic activity, which could account for the absence of astroglial cell death.

In conclusion, this thesis has discerned new non-apoptotic roles of caspase-3 in astroglial cells and suggests mechanisms for caspase-3 blockade in these glial cells, improving the understanding of the mechanisms employed by astrocytes to cope with damage in the immature brain.

RESUMEN

Español

Los mecanismos apoptóticos han sido estudiados en profundidad, constituyendo un papel substancial en la muerte neuronal tras diferentes tipos de daño en el SNC. No obstante, muy poco se sabe acerca de la activación de los mecanismos apoptóticos en las células gliales y su relevancia en la terminación de la respuesta glial. La caspasa-3 ha sido definida clásicamente como al principal ejecutora de la muerte celular programada, le han sido recientemente atribuidos otros papeles no-apoptóticos, participando en acontecimientos celulares, incluyendo proliferación celular, regulación del ciclo celular, y vías celulares apoptóticas y antiapoptóticas después de un daño excitotóxico en el cerebro inmaduro de rata. Para este propósito, se ha utilizado un modelo de excitotoxicidad bien caracterizado, que consiste en la inducción de un daño cerebral mediante una inyección intracortical de N-methyl-D-asparate (NMDA) en ratas postnatales en el día 9 de vida (P9). La muerte celular, las proteínas apoptóticas y antiapoptóticas fueron analizadas a distintos tiempos de supervivencia después de la lesión por ensayos enzimáticos y análisis de doble fluorescencia con microscopía confocal.

Los primeros resultados como la caspasa-3 clivada se presentaba en neuronas dañadas con los núcleos TUNEL+, aunque fue observada mayoritariamente en el núcleo de astrocitos activados en el hemisferio lesionado sin asociación con fragmentación del ADN, desde tiempos tempranos las 4 horas post-lesión (hpl) hasta la formación de la cicatriz glial a los 7-14 días, sin encontrarse asociación con marcaje con TUNEL. La actividad enzimática de la caspasa-3 fue detectada a las primeras horas post-lesión y coincidiendo con los fragmentos clivados por caspasas de la proteína acídica fibrilar (CCP-GFAP) en astrocitos. Sin embargo, a largos tiempos de supervivencia, cuando surge la hipertrofia astroglial, la caspasa-3 astroglial generalmente no correlacionó con el clivage de CCP-GFAP, pero en cambio se relacionó con la expresión de novo de vimentina. Por otra parte, la caspasa-3 clivada no se ha vinculado a proliferación celular. Estes resultados iniciales mostraron la evidencia de un no-tradicional papel de las caspasas en la función astroglial, sugeriendo que su activación puede ser importante para la reestructuración del citoesqueleto después de un daño.

Secundariamente, al evaluar las vías de activación de la caspasa-3 en neuronas y células gliales, la activación espacio-temporal de las vías apoptóticas intrínseca y extrínseca fueron analizadas. Se expresó principalmente en las neuronas dañadas corticales e hipocampales, una importante contribución de la vía intrínseca através de la gran proporción de caspasa-9 activa con la activación neuronal de la caspase-3 y muerte celular. Por otra banda, la caspasa-8 presentó una reducida correlación con la caspasa-3, y característicamente presentó con especificidad en las capas corticales. En las células gliales, la activación de la caspasa-9, caspasa-8, y también p53, otra molécula que activa la caspasa-3 indirectamente, fue solamente significante en la capa cortical VI y en el corpus callosum, sugeriendo que la caspasa-3 astroglial no se correlaciona generalmente con las clásicas vías de activación.

La tercera parte de esta tesis se focalizó en el estudio de las moléculas antiapoptóticas, como varios inhibitores de apoptosis (IAPs) y las heat shock proteins (HSPs), conocidas como inhibidoras de la caspasa-3 clivada en otros tipos celulares. Las neuronas presentaron una apreciable expresión de HSC70/HSP70 y cIAP-2, aunque estas pobremente se correlacionan con caspasa-3 en este tipo celular. En astrocitos , la survivina y la chaperona HSP25/27 mostraron una fuerte correlación con caspasa-3 en varias regiones cerebrales y a varios tiempos de supervivencia, siendo estas proteínas destacadas candidatas para el bloqueo de la actividad enzimática de la caspasa-3, la cual podría acumularse en ausencia de muerte astroglial.

En conclusión esta tesis ha destapado nuevos roles no apoptóticos de la caspasa-3 en células astrogliales, sugeriendo mecanismos de bloqueo de la caspasa-3, ayudando a la mejor comprensión de los mecanismos empleados por los astrocitos para enfrentarse al daño en el cerebro inmaduro.

RESUMO

Galego

Os mecanismos apoptóticos estudiáronse en profundidade, constituíndo un papel substancial na morte neuronal despois de diferentes tipos de dano no SNC. Nembergantes, moi pouco se sabe acerca da activación dos mecanismos apoptóticos nas células gliais e a sua relevancia na terminación da resposta glial. A caspasa-3 definiuse clásicamente coma principal executura da morte celular programada, fóronlle recentemente atribuídos outros papeis non-apoptóticos, participando en acontecementos celulais, incluíndo proliferación, regulación do ciclo celular, e vías apoptóticas e antiapoptóticas despois dun dano excitotóxico no cerebro inmaduro de rata. Para este propósito, utilizáronse un modelo de excitotoxicidade ben caracterizado, que consiste na inducción dun dano cerebral mediante unha inxección intracortical de N-methyl-D-asparate (NMDA) en ratas postnatais no día 9 de vida (P9). A morte celular, as proteínas apoptóticas e antiapoptóticas foron analizadas a distintos tempos de supervivencia despois da lesión por ensaios enzimáticos e análise de doble fluorescencia con microscopía confocal.

Os primeiros resultados coa caspasa-3 clivada presentábanse en neuronas dañadas cos núcleos TUNEL+, aunque foi observada maioritariamente no núcleo de astrocitos activados no hemisferio lesionado sen asociación con fragmentación do ADN, dende tempos máis cedos, ás 4 horas post-lesión (hpl) ata a formación da cicatriz glial ós 7-14 días, sen encontrarse asociación co marcaxe con TUNEL. A actividade enzimática da caspasa-3 foi detectada as primeiras horas post-lesión e coincidindo cos fragmentos clivados por caspasas da proteína acídica fibrilar (CCP-GFAP) en astrocitos. Sen embargo, a tempos longos de supervivencia, cando surxe a hipertrofia astroglial, a caspasa-3 astroglial xeralmente non correlacionou co clivaxe de CCP-GFAP, pero si se relacionou coa expresión de novo de vimentina. Por outra banda, a caspasa-3 clivada non se vinculou á proliferación celular. Estes resultados iniciais mostraron a evidencia dun non-tradicional papel das caspasas na función astroglial, suxerindo que a sua activación pode ser importante para a reestructuración do citoesqueleto despois dun dano.

Secundariamente, ó evaluar as vías de activación da caspasa-3 en neuronas e nas células gliais, a activación espacio-temporal das vías apoptóticas intrínseca e extrínseca foron analizadas. Expresouse principalmente nas neuronas dañadas corticais e hipocampais, unha importante contribución da vía intrínseca através da gran proporción de caspasa-9 activa coa activación neuronal da caspase-3 e morte celular. Por outra banda, a caspasa-8 presentou unha reducida correlación coa caspasa-3, e característicamente presentou especificidade nas capas corticais. Nas células gliais, a activación da caspasa-9, caspasa-8, e tamén p53, outra molécula que activa a caspasa-3 indirectamente, foi soamente significante na capa cortical VI e no corpus callosum, suxerindo que a caspasa-3 astroglial non se correlaciona xeralmente coas clásicas vías de activación.

A terceira parte desta tesis focalizouse no estudio das moléculas antiapoptóticas, coma varios inhibitores de apoptosis (IAPs) e as heat shock proteins (HSPs), coñecidas coma inhibidoras da caspasa-3 clivada noutros tipos celulares. As neuronas presentaron unha importante expresión de HSC70/HSP70 e cIAP-2, anque estas pobremente correlacionábanse coa caspasa-3 neste tipo celular. En astrocitos , a survivina e a chaperona HSP25/27 mostraron unha forte correlación con caspasa-3 en varias rexións cerebrais e a varios tempos de supervivencia, sendo estas proteínas destacadas candidatas para o bloqueo da actividade enzimática da caspasa-3, a cal podría acumularse en ausencia de morte astroglial.

En conclusión esta tesis destapou novos roles non apoptóticos da caspasa-3 en células astrogliais, suxerindo mecanismos de bloqueo da caspasa-3 e axudou á mellor comprensión dos mecanismos empregados polos astrocitos para enfrentarse ó dano no cerebro inmaduro.

RESUM

Catalá

S'han estudiat en profunditat els mecanismes apoptòtics, constituint un factor substancial en la mort neuronal després de diferents tipus de danys en el SNC. No obstant això, molt poc es sap sobre l'activació dels mecanismes apoptòtics en les cèl·lules glials i la seva rellevància en la terminació de la resposta glial. La caspasa-3 ha estat definida clàssicament com la principal executora de la mort cel·lular programada i li han estat atribuïts recentment altres papers no apoptòtics de participació en esdeveniments cel·lulars, incloent-hi la proliferació cel·lular, la regulació del cicle cel·lular, i la participació en vies cel·lulars apoptòtiques i antiapoptòtiques després d'un dany excito-tòxic en el cervell immadur de rata. Per aquest propòsit, s'ha utilitzat un model de excitotoxicitat ben caracteritzat, que ha consistit en la inducció d'un dany cerebral mitjançant una injecció intracortical de N-methyl-D-asparate (NMDA) en rates postnatals en el 9è dia de vida (P9). La mort cel·lular, les proteïnes apoptòtiques i antiapoptòtiques van ser analitzades a diferents terminis de supervivència després de la lesió per assaigs enzimàtics i anàlisis de doble fluorescència amb microscopia confocal.

Els primers resultats com la caspase-3 clivada es presentava en neurones danyades amb els nuclis TUNEL+, encara que va ser observada majoritàriament en el nucli d'astròcits activats en l'hemisferi lesionat sense associació amb fragmentació del ADN, des de les 4 hores post-lesió (hpl) fins a la formació de la cicatriu glial als 7-14 dies, sense trobar-se associació amb marcatge amb TUNEL. L'activitat enzimàtica de la caspasa-3 va ser detectada a les primeres hores post-lesió i coincidint amb els fragments clivados per caspasas de la proteïna acídica fibrilar (CCP-GFAP) en astrocitos. No obstant això, sobre llargs terminis de supervivència, quan sorgia la hipertròfia astroglial, la caspasa-3 astroglial generalment no es va correlacionar amb el clivage de CCP-GFAP, però en canvi es va relacionar amb l'expressió *de novo* de vimentina. Per altra banda, la caspasa-3 clivada no s'ha vinculat a proliferació cel·lular. Aquests resultats inicials van mostrar l'evidència d'un paper gens tradicional de les caspases en la funció astroglial, suggerint que la seva activació pot ser important per a la reestructuració del citoesquelet després d'un dany.

Secundàriament, al avaluar les vies d'activació de la caspasa-3 en neurones i cèl·lules glials, l'activació espai-temporal de les vies apoptòtiques intrínseca i extrínseca va ser analitzada. Es va expressar principalment en les neurones danyades corticals i hipocampals, una important contribució de la via intrínseca a través de la gran proporció de caspasa-9 activa amb l'activació neuronal de la caspase-3 i mort cel·lular. Per altra banda, la caspasa-8 va presentar una reduïda correlació amb la caspasa-3, i característicament es va presentar amb especificitat en les capes corticals. En les cèl·lules glials, l'activació de la caspasa-9, caspasa-8, i també p53, altra molècula que activa la caspasa-3 indirectament, va ser significant tant sols en la capa cortical VI i en el corpus callosum, suggerint que la caspasa-3 astroglial no es correlaciona generalment amb les clàssiques vies d'activació.

La tercera part d'aquesta tesi es va focalitzar en l'estudi de les molècules antiapoptòtiques, com diversos inhibidors de l'apoptosis (IAPs) i les proteïnes heat shock (HSPs), conegudes com inhibidors de la caspasa-3 clivada en altres tipus cel·lulars. Les neurones van presentar una apreciable expressió de HSC70/HSP70 i cIAP-2, encara que aquestes es correlacionaven pobrament amb caspasa-3 en aquest tipus cel·lular. En astròcits , la survivina i la chaperona HSP25/27 van mostrar una forta correlació amb caspasa-3 en diverses regions cerebrals i a diversos terminis de supervivència, sent aquestes proteïnes destacades candidates per al bloqueig de l'activitat enzimàtica de la caspasa-3, la qual podria acumular-se en absència de mort astroglial.

En conclusió, aquesta tesi ha descobert nous rols no apoptòtics de la caspasa-3 en cèl·lules astroglials i suggereix mecanismes de bloqueig de la caspasa-3, ajudant al coneixement dels mecanismes emprats pels astròcits per enfrontar-se al dany en el cervell immadur.

1. INTRODUCTION

1.1. THE IDENTITY OF APOPTOTIC CELL DEATH

Programmed cell death was first described by Richard Lockshin in 1964 through his observations about insect embryogenesis [1, 2]. Apoptosis, a term derived from the Greek words apó (from) and ptósis (fall), a reference to the falling of leaves from trees, was coined by Kerr in 1972 [3]. In 1986, Robert Horvitz established the molecular basis of this phenomenon. He reported specific cell death during the development of the nematode *Caenorhabditis elegans* and cloned the genes responsible for cell death (CED genes) [4]. These studies led Horvitz to win the Nobel Prize in 2002. Stimulus causes the cells to respond to several processes which end in death, described as either apoptotic, non-apoptotic cell death, or necrosis (for review see [5]).

Apoptosis is considered a process of cell *suicide* that is regulated by complex molecular signalling systems that trigger orderly, energy-dependent enzymatic breakdown of DNA, lipids and other macromolecules. Throughout this process the cell morphology suffers several changes: the chromatin is condensed, the nucleolus is disintegrated and the nucleus weight is reduced. The cell undergoes swelling and grows denser, the cytoplasmatic organelles are condensed and the endoplasmatic reticulum is dilated, but the mitochondria do not change morphologically [5]. The nucleus is fragmented and packaged into vesicles, called apoptotic bodies, which may be phagocytosed and reused. In the central nervous system (CNS) apoptotic cell death plays a key role in brain development and in neurodegenerative disease, trauma, ischemia and other types of acute CNS damage.

1.2. ACTIVATION OF APOPTOTIC PATHWAYS

1.2.1. Caspase-dependent cell death.

Caspases are aspartic acid-specific cysteine proteases, which become activated in most forms of cell death. In the cell, caspases are localized in the nucleus, cytoplasm, endoplasmatic reticulum, and mitochondrial intermembrane space, and they can be translocated to the plasma membrane [6, 7]. Currently, fourteen mammalian homologues of CED-3 have been cloned, characterized, and named caspases 1 to 14. They can be divided into two blocks, one comprising inflammatory caspases (caspases-1, -4, -5, -11, -12, -13, and -14), being primarily involved in procytokine activation [8], and the other comprising caspases involved in apoptosis, both initiator and effector caspases (caspases-2, -3, -6, -7, -8, -9, -10, and -12) (Table 1). After a cell receives an apoptotic signal, sequential activation of these caspases takes place; i.e. an upstream initiator caspase processes and activates downstream effector caspase(s) (Table 1). By this mechanism an apoptotic signal gets amplified rapidly, as proteolytic activation of pre-existing molecules occurs instead of a much more slowly de novo-synthesis.

Group I: **Inflammatory caspases**	Caspase-1
	Caspase-4
	Caspase-5
	Caspase-11
	Caspase-12
	Caspase-13
	Caspase-14
Group II: **Apoptosis initiator caspases**	Caspase-2
	Caspase-6
	Caspase-8
	Caspase-9
	Caspase-10
	Caspase-12
Group III: **Apoptosis effector caspases**	Caspase-2
	Caspase-3
	Caspase-6
	Caspase-7

Table 1. *The caspase family. Group I: inflammatory caspases; Group II: apoptosis initiator caspases; Group III: apoptosis effector caspases.*

1.2.2. Procaspases and their activation.

Caspases are present in cells as zymogens and they need to undergo proteolytic cleavage in order to achieve their enzymatic activity. Caspases are expressed in most tissues as inactive proforms, which have an amino-terminal pro-domain (23 to 219 kDa), a large subunit (~20 kDa), and a small subunit (~10 kDa). Prior to activation, both subunits dimerize with another cleaved caspase molecule and form an active heterotetrameric structure. Upon activation, procaspases are proteolytically cleaved at the carboxyl side of aspartate resides (Asp-X) to remove the pro-domain and release the large and small subunit [9, 10], which are assembled together to yield an active heterotetrameric caspase enzyme [11] (Figure 1). Two related motifs in the N-terminal region of initiator caspases have been identified: the death effector domain (DED), and the caspase recruitment domain (CARD). The DED is found in caspase-8 and -10, and CARD is found in caspase-1, -2, -4 and -9.

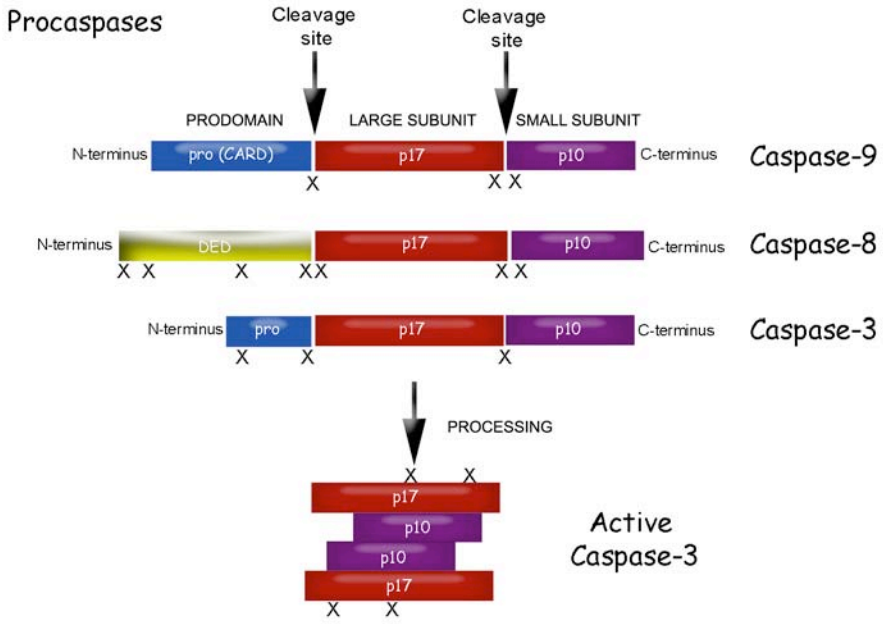


Figure 1*. Caspase structure and activation. Scheme of cleavage of the procaspases (-8 and -9) at the specific Asp-X bonds leads to the formation of the active caspase-3. The* Caspase recruitment domains (*CARD), the* Death Effector Domains (*DED), and the large (p17) and small (p10) catalytic subunits with N and C termini are indicated.*

The active site consists of residues from both; thus, an active caspase molecule has two sites for substrate binding and cleavage. Initiator caspases are activated by oligomerization and conformational changes but do not require cleavage for activation. Executioner caspases require cleavage to the p10/p20 peptide fragments to assemble and form activated enzymes [12, 13].

1.2.3. Caspase cascades: two pathways.

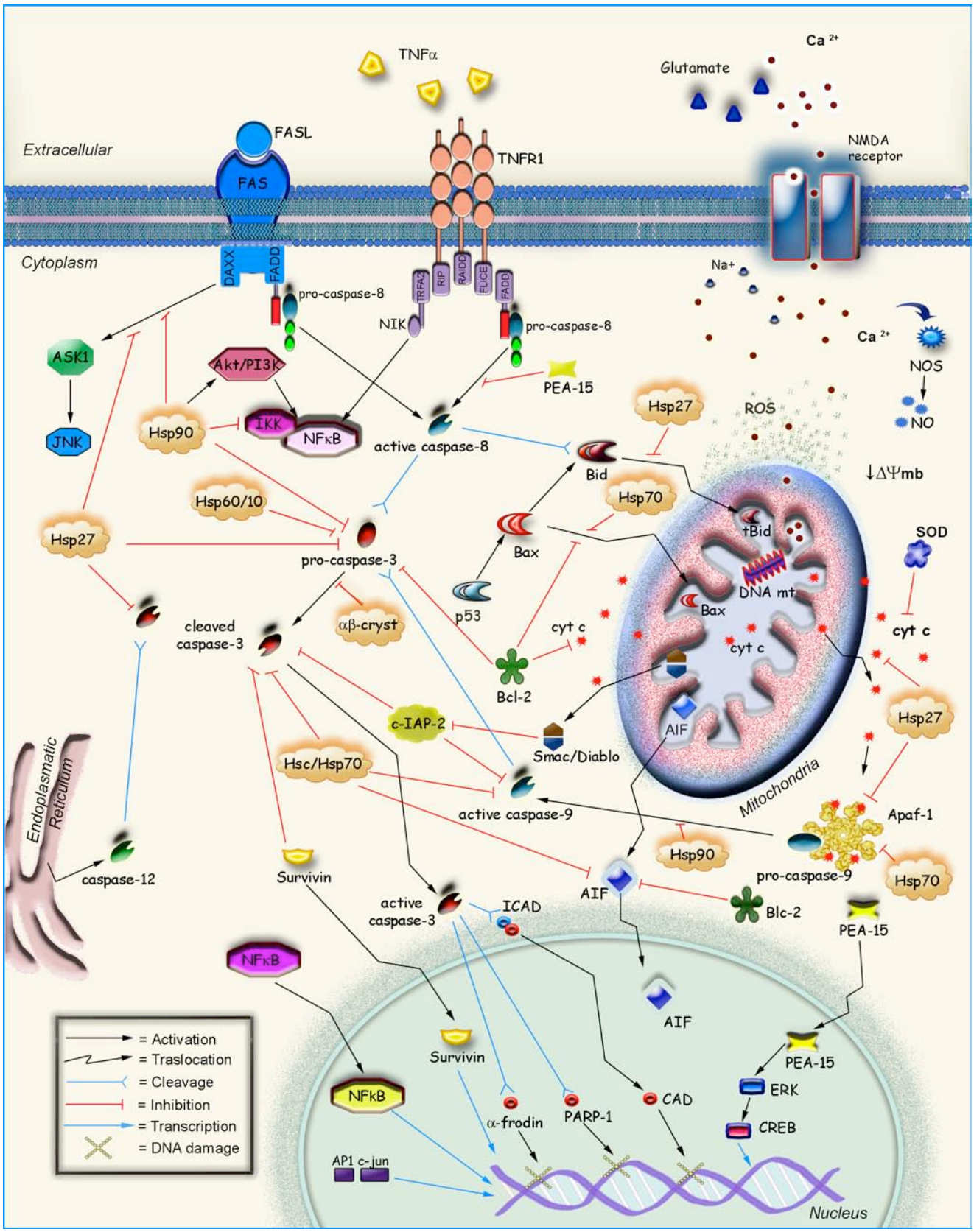
Two main pathways of apoptosis signalling have been well documented in the mammalian cells; the "extrinsic" or death receptor-initiated pathway, and the "intrinsic" or mitochondrial pathway [14]. A schematic representation of both pathways in response to different stimulus is illustrated in Figure 2.

1.2.3.1. Mitochondrial pathway or Intrinsic Pathway.

Mitochondria plays an important role in the execution process of the apoptotic program by acting as a reservoir for a multitude of apoptogenic proteins [15-17]. After release from the mitochondria, cytochrome c associates with the apoptosis protease activating factor-1 (Apaf-1), 2′-deoxyadenosine 5′-triphosphate (dATP), inducing a conformational change and triggering its oligomerization and the subsequent recruitment and autoprocessing of inactive procaspase-9 to form the apoptosome complex, hence initiating downstream caspase activation [18] (Figure 2). Activation of caspase-9 precedes the maturation and activation of effector caspase-3 [19].

1.2.3.2. Death receptors pathway or Extrinsic Pathway.

Several extrinsic ligands can activate "death receptors" of the tumor necrosis factor receptor (TNFR) family, including: apoptosis antigen-1 (APO1/FAS/CD95), or tumor necrosis factor receptor-1 (TNFR1) (Figure 2). The FAS ligand (FASL/CD95L) is a member of the TNF super family of cytokines, and along with its receptor FAS, associate to inflammatory and immune responses in the CNS [20]. FASL-mediated activation of FAS leads to the recruitment of FAS-associated death domain (FADD) and promotes caspase-8 activation [21, 22] (Figure 2). Several caspase-8 molecules cause auto-activation and processing of caspase-8 molecules which are then ready to activate downstream caspases [23-26], resulting in cleavage of Bid to truncated Bid (tBid), a pro-apoptotic member of the Bcl-2 family.



Apoptotic and antiapoptotic mechanisms in neurons and glial cells after damage to the immature brain.

Figure 2. *Proapoptotic and antiapoptotic signalling.* Oxidative stress leads to the loss of mitochondrial membrane potential and the release of cytochrome c, which involves the formation of Apaf-1, leading to the activation of the intrinsic pathway via activation caspase-9 and effector caspase-3. In addition, ligand binding to fibroblast-associated cell surface (FAS) or tumor necrosis factor receptor-1 (TNFR1) stimulate the extrinsic pathway in a series of steps leading to the cleavage and activation of caspase-8, which activates effector caspase-3. Caspase-8 can also mediate BH3-interacting domain (Bid) cleavage, resulting in translocation of truncated bid (tBid) to mitochondria providing a link between intrinsic and extrinsic pathways. Key regulating proteins, such as apoptosis-inducing factor (AIF), Bcl-2 family proteins, the inhibitor of apoptosis protein (IAP), and heat shock proteins (HSP) converge upon activation/inhibition of caspases and apoptotic cascades.

1.2.4. Main executor: Caspase-3.

Caspase-3 is a principal member of the cysteine protease family that is in charge of the execution of apoptotic cell death. It appears to be the most abundant of the known caspases in the brain, the most studied, and the convergence of all caspase-mediated pathways related to apoptosis. It is activated by both intrinsic and extrinsic cell signalling pathways, by proteolytic cleavage into p17 and p12 subunits, which heterodimerize to form the active enzyme [27]. During normal brain development, when more than half of the neurons in some brain regions are removed through apoptosis, caspase-3 is highly upregulated, playing an essential role. Procaspase-3 is highly expressed from E17 (17th embryonic day) to P7 (7th postnatal day), decreasing after P14 (14th postnatal day) in the rat [28, 29]. The importance of caspase-3 activation both in normal CNS development and after damage comes from studies using specific inhibitors or genetically modified animals. As a result, caspase-3 null mice display considerable neuronal expansion and unusually abnormal brain development, resulting in death by the second week of life [30, 31]. In addition, specific inhibitors of this caspase have been shown to be neuroprotective in different neurodegenerative conditions [32, 33].

1.2.5. Balance between proapoptotic and antiapoptotic molecules.

Apoptotic factors activated after an insult are usually accompanied by overexpression of molecules aimed to inhibit them: the antiapoptotic proteins. The balance between concentrations of both groups will finally determine if the cell will die or survive. Therefore, final effects of apoptotic caspase cascades cannot be studied independently form antiapoptotic mechanisms and caspase-independent proapoptotic pathways concomitant within the same cell.

The **family of proapoptotic and antiapoptotic Bcl-2 proteins** located on the surface of mitochondria appears to play a major role in the release of proapoptotic proteins and in regulating cell death. The Bcl-2 family consists of several main subgroups, which are categorized according to their anti- or pro- death function, and the presence or absence of conserved structural motifs - the Bcl-2 homology (BH) domains. The antiapoptotic group includes Bcl-2, Bcl-xs, Bcl-w and myeloid cell leukaemia-1 (MCL1). The proapoptotic group includes B-cell lymphoma 2-associated protein X (Bax), Bcl-2 agonist killer 1 (Bak), Bcl-2-related ovarian killer protein (Bok) and Bcl-xl, all of which have at least BH1 and BH3 domains. Bax is essential for apoptotic death in most developing neurons and exists in a stimuli-dependent equilibrium between an inactive soluble cytosolic form and an active mitochondrial membrane-associated form that induces cytochrome c release and subsequent activation of the execution phase of apoptosis.

The **tumor suppressor p53** acts in response to a variety of cellular stresses to interfere with cellular processes including cell cycle arrest, DNA repair, senescence and differentiation [34-36]. P53 is also important in the control of apoptosis as it can engage both intrinsic and extrinsic apoptotic pathways (Figure 2), mainly through transcriptional regulation of a growing number of target genes including the proapoptotic Bcl-2 members Bax, PUMA, Apaf-1 and Noxa (Table 2) involved in the mitochondrial pathway, and the death receptors TRAIL-R1 and FAS of the extrinsic pathway (Figure 2).

Apoptosis-inducing factor (AIF) is a mitochondrial intermembrane flavoprotein with proapoptotic effects. It traslocates from mitochondria to nucleus in a caspase-independent pathway, causing DNA fragmentation [19], usually earlier than the release of cytochrome c. **Smac/DIABLO** is released from the mitochondria along with cytochrome c [37] and it binds the inhibitors of apoptosis (IAPs) and disengages them from activated caspases, thus promoting caspase activity and enabling the execution of apoptosis. In fact, the balance between IAPs and Smac/DIABLO establishes a threshold for caspase-3 activity [38].

Another pathway involved in stress response and apoptosis is mediated by **mitogen-activated protein kinases (MAPK),** a family of proteins that has three major members: extracellular signal-

regulated kinase (ERK), c-Jun N-terminal kinases (JNKs) and p38 MAP kinase (p38), playing a significant role in neuronal survival or death. ERK is activated by growth factors or mitogens leading to proliferation, differentiation, and survival via activation of neurotrophins, such as the brain-derived neurotrophic factor (BDNF) [39]. On the other hand, JNK and p38 are preferentially activated by oxidative stress and cytokines resulting in inflammation and apoptosis through activation of mitochondrial related proapoptotic proteins [40].

Finally, **nuclear factor kappaB (NFkB)** has been found to respond to a broad range of stimuli and conditions, including inflammatory cytokines, growth factors, adhesion molecules, cell surface receptors, extracellular stress and intracellular oxidative stress. NFkB is crucial for inflammatory reactions in the periphery and regulates expression of both apoptotic and antiapoptotic genes (Figure 2).

Pro-apoptotic Molecules		Anti-apoptotic Molecules	
AIF	[19, 41-44]	Akt	[45-49]
Bad	[50]	Bcl-2	[51-54]
Bak	[55]	Bag-1	[56]
Bax	[57-64]	Bcl-xl	[65, 66]
Bid	[67]	Bcl-w	[68]
Bim	[69]	ERK	[70-73]
JNK	[74-78]	MAPK	[79-81]
Noxa	[82, 83]	NFkB	[84, 85]
p38MAK	[86]	PEA-15	[87-91]
p53	[34, 83, 92-94]	PI3K	[95, 96]
Puma	[97]		

***Table 2**. Several Pro-apoptotic and Anti-apoptotic molecules.*

1.2.6. Main caspase substrates.

The latest results describing caspase-mediated cleavage events are: 1) to stop cell-cycle progression, 2) breakdown of the structural components of the cell, 3) impairment of homeostatic and repair mechanisms, and 4) cell detachment from the surrounding tissue. Caspases have an executioner role in death mechanisms via the breakdown of cellular framework through activation, cleavage, and degradation of different substrates (Table 3; left). Executor caspases cleave several intracellular membranes, and nuclear proteins, with a preference for the DEVD/DXXD sequence [98], inducing disintegration of nucleolar structure and Poly (ADP-Ribose) Polymerase-1 (PARP-1) cleavage, separating the DNA-binding domain, essential for the stimulation of the enzyme, and the catalytic domain [99, 100]. Some caspases have common substrates (caspase-3 and caspase-7 both cleave PARP), whereas others are more selective (protein kinase C δ (PKCδ) is cleaved by caspase-3, but not by caspase-7), or have unique substrate specificities (caspase-6 is the only caspase known to cleave lamins). An important mediator of cell cycle progression, the retino blastoma protein (Rb), is also proteolytically cleaved during apoptosis [101]. To date, the list of known caspase substrates includes almost 300 targets that belong to a variety of functional groups (summarized in Table 3), not only well known proapoptotic substrates, but also other cell components suggesting that caspases might also have additional non-apoptotic roles (see below).

1.2.7. Non-apoptotic roles of caspases.

Recent studies have suggested that activated caspases may also be important for several non-apoptotic processes (Table 3; right columns, reviewed in [103, 104, 106, 108, 109]. In general, caspases are now known to mediate cleavage of substrates that may be involved in the regulation of apoptosis-independent processes such as proliferation, regulation of cell cycle, activation of survival pathways, spreading, migration, differentiation, maturation, receptor internalization, and inflammation [104, 108]. Caspase-1 has long been identified as the protease involved in IL-1 cytokine maturation. Particularly, active caspase-3 has been involved in cellular processes such as cell differentiation, migration, or plasticity [110, 111] (Table 3), by regulating cytoskeletal components such as actin, gelsolin, vimentin, cytokeratin, and the astroglial GFAP, suggesting that caspases could potentially modulate the function of some of these proteins and thus regulate cytoskeletal-mediated migration [112, 113].

Caspase Substrates

	Proapoptotic substrates	Non-apoptotic substrates	Associated cellular process
Caspase-1	Calpastatin, procaspase-3/4, Bcl-X_L	Pro-interleukin-1β	Cytokine maturation
		Pro-interleukin-18	Cytokine maturation
Caspase-3	*DNA cleavage and repair*	Acinus	Erythrocyte differentiation, enucleation
	PARP, Acinus, DNA-PKcs, DFF, ICAD, Topoisomerase 1	Calpastatin	Myoblast differentiation
		EAAT2	Astrocyte glutamate transporter
	Cytoskeletal and structural proteins	GATA-1	Eryrthopiesis regulation
	Vimentin, actin, GFAP, Frodin (alpha II-spectrin), Lamin A, Lamin B1, Gelsolin, β II-spectrin, β-Catenin, cytokeratin 18	Gelsolin	Outgrowth and cell spreading
		GFAP	Cytoskeletal remodeling
		GLUR1 / GLUR4	Glutamate transport
	Protein kinases of signal transduction	FAK	Cytoskeletal regulation
	Protein phosphatase 2A subunit Aα, PKCτ, PKC-related kinase, PKN, PITSLRE kinase α2-1, MEKK-1, CaMK IV, Pro-interleukin-16, D4-GDP dissociation inhibitor, Cytosolic phospholipase A_2	FLIPL	T and B cell activation
		FOXO3a	Proliferation
		Lamin B	Erythrocyte differentiation
	Transcription	Mst1/2 kinase	Myoblast differentiation
	Stat1, NFκB p50, p65, IκB, Ring1B, Steroid response element-binding proteins	$p21^{clp1/waf1}$, p27kip1	Cell cycle regulation
		p35	Caspase inhibition
	Cell cycle and Replication	PAK2	Cytoskeletal regulation, morphogical changes
	Replication Factor C large subunit, NuMA, HnRNP proteins C1 and C2	PARP-1	Erythrocyte differentiation, Lens fiber formation, T and B cell activation, NFkB activation
	Others	PKCδ	Keratinocyte differentiation, TCR signalling
	Persenilin-1, Bcl-2, Bcl-X_L, $FLIP_L$, Bid, Bax, DCC, p53, Cdc5, Huntington, Parkin, Persenilin-2, Dentatorubral pallidalysian atrophy protein	PKCζ	Cell growth signalling
		Plectin	Cell spreading
		PLA2	ROS decrease
		Rb protein (retinoblastoma)	Cell cycle regulation
		RasGAP	Morphogical changes, adhesion, cytoskeletal regulation
		SLK	Cytoskeletal regulation, actin assembly
		Vimentin	Cytoskeletal remodeling
		Wee1	Cell cycle regulation

		FAK	Cytoskeletal regulation
Caspase-6	Cytokeratin 18, Lamin A	$p27^{kip1}$	Cell cycle regulation
		PKCθ	TCR signalling
		FAK	Cytoskeletal regulation
Caspase-7	Cytokeratin 18, Mdm2, PARP, proteins, calpastatin, Steroid response element-binding, Huntington, Hn-ribonuclear protein C	$p21^{waf1}$, $p27^{kip1}$	Cell cycle regulation
		PKCθ	TCR signalling
		TCR ζ chain	TCR signalling
		Wee1	Cell cycle regulation
Caspase-8	RIPK1, FLIP, Procaspases-3, -4, -7, 9	PAK2	Cytoskeletal regulation
		Plectin	Cell spreading
		Wee1	Cell cycle regulation
		PKCθ	TCR signalling
Caspase-9	PARP, Procaspase-3	SRPK2	RNA splicing
		TCR ζ chain	TCR signalling

Table 3*. Caspases and their substrates in apoptotic leading to apoptosis and non-apoptotic processes. Based on the following reviews: [8, 102-107].*

In addition, in the brain, caspase-3 has been implicated in preconditioning. McLaughlin and coworkers postulated that preconditioning induces cellular resistance through a caspase-3 mediated HSP70 upregulation mechanism, preserving the integrity of the mitochondrial membrane and acting downstream of caspase-3 activation to inhibit cell death [114-116]. In some instances, caspases can also activate survival pathways, such as the NFκB pathway [104]. However, caspase-8 was shown to interact with RIP, NFκB-inducing kinase (NIK) and IKKs, resulting in increased kinase activity of the IKK-signal complex [104]. Caspases may play a role in the regulation of the cell cycle by serving as additional checkpoints to ensure that only healthy cells complete the cell cycle (Table 3; right columns). An example in the arrest of cell cycle by action of several caspases is the cleavage of the Rb, mdm2, Wee1, cdc2, cdc27 and cyclin E. In addition, $p21^{CIP1/WAF1}$ and $p27^{kip1}$ are cleaved by caspase-3, caspase-7, and caspase-8 and obstruct the activation of cyclin-cdk2 complexes, preventing progression from G1 to S

phase [117, 118]. Similarly, several reports have investigated the role of caspases in the induction of proliferation [109] or differentiation [119]. Caspase-8 regulates T cell proliferation through T cell receptor (TCR) stimulation [85] and a defect in the production of IL-2 by means of an unknown mechanism.

1.3. INHIBITORY MOLECULES THAT BLOCK CELL DEATH.

1.3.1. Inhibitor of apoptosis proteins: IAPs.

Caspase proenzymes and active forms are controlled, under normal physiologic conditions, by endogenous inhibitors such as inhibitor of apoptosis proteins (IAPs) or the Heat Shock Proteins (HSPs). IAPs are a family of pro-survival genes that act downstream of caspase activation to directly bind and inhibit activated caspases regulating their degradation at the level of the proteasome [120]. The functional basis of IAPs are the baculoviral IAP repeat (BIR) domains through which they bind to and inhibit caspases [121] (Figure 3).

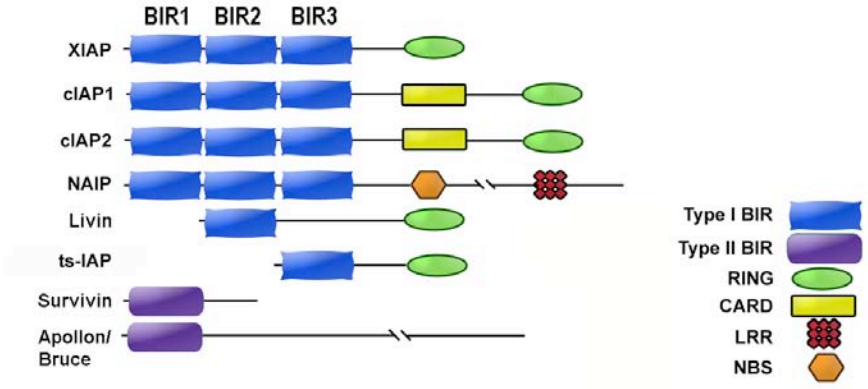


Figure 3. *Mammalian IAPs. BIR, baculovirus IAP repeat; RING, RING finger domain; CARD, caspase-recruitment domain; LRR, leucine-rich repeats; NBS, nucleotide binding site. (Modified from [122]).*

Overall, there are two general types of BIR domains: Type I domains that bind to and inhibit caspases; and Type II domain, which can also bind caspases, but act on the cell cycle as well (Figure 3). Type I BIR domains are classified into: BIR1, -2, and -3. Binding of the linker region between BIR1 and BIR2 inhibits executor caspases-3 and -7, and BIR3 binds to and inhibits caspase-9. The BIR1 domain has not caspase-inhibiting activity. Four of the mammalian IAPs: cellular inhibitor of apoptosis

(cIAP)-1, cellular inhibitor of apoptosis (cIAP)-2, X-Chromosome-Linked Inhibitor of Apoptosis Protein (XIAP), and Neuronal Apoptosis Inhibitory Protein (NAIP), contain all three BIR type I domains; the other four IAPs, survivin, livin/ML-IAP, APOLLON/BRUCE, and testicular IAP (ts-IAP), contain either only one BIR domain or type II BIR domains, but they can also inhibit caspases-3/-7 and -9 [122, 123]. In this regard, final cleavage of executor caspase-3 substrates does not only depend on caspase activation, but also on the presence or not of specific IAPs that can block apoptotic pathways downstream of effector caspase activation. In this sense, XIAP, cIAP-1, cIAP-2, NAIP and survivin have been shown to inhibit caspase-3 in peripheral organs but also in the CNS, where several IAPs such XIAP, cIAP-1 [122], NIAP [124] or survivin [125] have been show to provide neuroprotection against ischemia [126], trauma [127], and other neurodegenerative paradigms, mainly through caspase-3 inhibition (Table 4). In addition, cIAP-2 has been shown to be one of the main factors contributing to neuroprotection in preconditioning [116]. There is not yet a clear consensus on the function of the different IAPs in the immature brain, although it is known that IAPs are much more frequent in the neonate, and its overexpression is neuroprotective. Knockout of survivin is embryonically lethal; embryos survive only 5 days due to cytokinesis failure [128].

Endogenous Inhibitors of Caspase-3	Cell Type in CNS	References
XIAP	Neurons, oligodendrocytes	[38, 116, 126, 129-134]
cIAP-1	Neurons, astrocytes, Neural progenitor cells	[131, 135, 136]
cIAP-2	Neurons, oligodendrocytes, astrocytes	[116, 131, 134, 137]
NAIP	Neurons	[124, 133, 138-141]
Survivin	Neurons, astrocytes, Neural progenitor cells	[127, 128, 141, 142]
HSP25/27	Neurons, astrocytes	[143-151]
HSC70/HSP70	Neurons, oligodendrocytes, astrocytes	[152, 153]
HSP70/72	Neurons, astrocytes	[114, 146, 154-156]
Hsp110	Neural crest cells	[157]

Table 4. *Endogenous Inhibitors of caspase-3 activation in the CNS.*

1.3.2. Heat Shock Proteins and the stress response.

HSPs function as molecular chaperones and are crucial for recovery from stress-induced protein damage increasing survival (for review [158]). An important function of HSPs is to act as protein chaperones mediating transport of proteins into organelles such as lysosomes and endoplasmic reticulum or folding nascent polypeptides during translation [159]. In response to stress, HSPs are induced and they prevent formation of protein aggregates and contribute to the stabilization of denatured proteins and the removal of damaged proteins for degradation, as well target them for destruction by the proteasome both actions serving a protective role [160]. HSPs are classified into several multi-gene families based on their amino acid sequences, functions and molecular weights from 10 to 174 kD. They are divided into families of HSP27, HSP40, HSP60, HSP70, HSP90 and HSP110 with several functions in the cells (Table 5).

HSP Families	Chaperones or isoforms	Subcellular location	Apoptotic proteins chaperoning	References
HSP27	αβ-crystallin	Cytosol	Pro-caspase-3, Bax, Bcl-Xs, cytochrome c, caspase-8	[161, 162]
	HSP20	Cytosol/nucleus	Pro-caspase-3	[163]
	HSP25/27	Cytosol/nucleus	Procaspase-9, cytochrome c, procaspase-3, tBid, DAXX, Akt, IkB	[147, 148, 164-166]
HSP40	HSP47	Cytosol, endoplasmic reticulum (ER)	Caspase-3, CHOP, Bax	[149, 153, 167]
HSP60	HSP60/HSP10-cyt	Cytosol	Pro-caspase-3, active caspase-3, Bax	[162, 168]
	HSP60/HSP10-mit	Mitochondria	Pro-caspase-3, Bcl-Xl cytochrome c	[169]
HSP70	HSC70	Cytosol/nucleus	Caspase-3	[153, 170-172]
	HSP71	Cytosol	Caspase-3	[155]
	HSP72	Cytosol/nucleus	Caspase-3, Apaf-1, Fas	[156, 173-175]
	HSP70	Cytosol/nucleus/Mitochondria	Caspase-3, JNK, AIF, CHOP, Bax, apoptosome, SEK kinase, Bid	[154, 159, 167, 176-181]
	Grp78 (Bip)	ER/Cytosol/nucleus	Caspase-12, CHIP	[140, 182-184]
HSP90	HSP90-α	Cytosol/nucleus	Apaf-1, ASK-1, IKK, Akt	[150]
	HSP90-β	Cytosol/nucleus	NF-kB	[150]
	Grp94	ER	Pro-caspase-3, active caspase-3	[185]
HSP110	HSP105	Cytosol/nucleus	Pro-caspase-3, p38MAK, cytochrome c	[186, 187]
	HSP110/104	Cytosol/nucleus	Pro-caspase-3, caspase-9	[157]

Table 5. *Mammalian HSPs: Nomenclature, subcellular location, and proapoptotic proteins which they chaperone (taken from [158, 188, 189] and [190]).*

Under normal conditions, certain HSPs are constitutively present and are named Heat Shock Cognates (HSC) which are essential for the folding of nascent proteins and for protein translocation and for maintaining multiprotein complexes.

HSP27 belongs to the family of small stress proteins that are constitutively abundant and interacts with key elements of the apoptotic-signalling pathways. HSP27 blocks DAXX-mediated apoptosis and inhibits its interaction with Fas and ASK-1, also prevents translocation of tBid onto the mitochondrial membrane, regulating apoptosis through interaction with Akt (Figure 2). In addition, HSP27 stabilizes the cytoskeleton as the proteolytic cleavage of actin by caspases has been proposed to have a role in the specific and sequential changes of different regulators of the microfilament system during the apoptotic process [164]. Overexpression of HSP27 was shown to reduce oxidative DNA damage by maintaining the redox homeostasis and mitochondrial stability, as it can sequester cytochrome c and procaspase-3 [148]. Aβ-crystallin, another small HSP of the same family, interferes in the processing of procaspase-3 precursor or it binds to the cleaved p24 fragment of caspase-3 before in undergoes maturation into a p20 subunit, and it can also mask Bax and Bcl-xs in the cytoplasm [161]. Mitochondrial HSP60 and its co-chaperone HSP10 are chaperone proteins responsible for the folding of newly imported proteins encoded by mitochondrial DNA, and they are associated with procaspase-3. HSP60 enhances its protease-sensitivity and thus makes it more susceptible to the action of cytochrome c [168, 169]. The HSP70 family is the most diverse, both constitutively and under stress conditions, acting as antioxidant against ROS [171]. HSP70 is also known to antagonize apoptosis by binding to AIF released from the mitochondria [191] or preventing the formation of the apoptosome by binding to Apaf-1 and blocking the activation of caspase-9 or caspase-3 and the appearance of apoptosis after DNA damage [192]. Overexpression of HSP70 inhibits the translocation of Bax from the cytoplasm to the mitochondria and it protects the cells from caspase-3 activation [154, 193]. HSP90 blocks apoptosis through its involvement at different steps in the formation of active IKK or Akt complexes and the dissociation of NF-κB from its inhibitor [194]. HSP90 and Akt promote cell survival by inhibition of JNK pathway through phosphorylation and consequent inactivation of ASK-1. In addition, HSP90 participates in the inhibition of Apaf-1 and in the formation of an inactive apoptosome complex [190] (Figure 2 and Table 5).

Although HSP actions are not exclusive to the nervous system, in the brain overexpression of HSP family members is neuroprotective against glutamate toxicity and oxidative injury and they provide several neuroprotective mechanisms [160, 193].

1.4. PROAPOPTOTIC AND ANTIAPOPTOTIC MECHANISMS IN EXCITOTOXIC CELL DEATH

Excitotoxicity is caused by pathological overactivation of glutamate receptors and is considered to be one of the underlying mechanisms of neuronal death in hypoxia, ischemia, seizures and hypoglycemia, and also in progressive neurodegenerative diseases [195]. In physiological conditions, glutamate is the major excitatory neurotransmitter in the mammalian brain and a key mediator of intracellular communication, plasticity, growth and differentiation [196], acting via metabotropic (mGluR) and ionotropic receptors (iGluR). The ionotropic glutamate receptors are the major mediators of excitotoxicity and, according to their differential agonist specificities, are named NMDA (N-methyl-D-aspartate) receptor, AMPA (amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptor, and KA (kainate) receptor [197]. In this sense, several glutamate agonists like NMDA, KA and AMPA have been used in the last decades as models of CNS neurodegeneration. Ionotropic receptors act as ligand-gated Ca^{2+} channels and after prolonged activation intracellular Ca^{2+} increases to concentrations that are sufficient to trigger downstream processes that ultimately result in cell death [198] (Figure 2). Ca^{2+} enters the cell and concomitant Na^{+} uptake is followed by an influx of chloride and water causing rapid neuronal swelling and activation of various cascades including activation of catabolic enzymes such as proteases, lipases and endonucleases, impairment of energy metabolism, decrease of adenosine triphosphate (ATP), membrane depolarization [199], and generation of ROS, causing oxidative stress that leads to the breakdown of the cell membrane, the cytoskeleton, the genomic DNA, and ultimately causes cell death (Figure 4). ROS particularly responsible for oxidative stress include: O_2^{-}, $OH^{\cdot}$, hydrogen peroxide (H_2O_2), NO and $ONOO^{-}$ (peroxynitrite). NO produced by activation of the neuronal isoform of nitric oxide synthase (nNOS) in response to NMDA receptor stimulation, as well as from activation of inducible NOS (iNOS), has been shown to be associated with mitochondrial dysfunction. Whereas high concentrations of excitatory aminoacids induce necrotic cell death very rapidly, lower

concentration of excitotoxins elicit apoptosis in a more delayed time course as it occurs in the ischemia penumbra or lesion border in other acute CNS injuries. However, as previously stated, cell fate does finally depend on the balance between apoptotic mechanisms and survival factors [200] (Figure 4). Proapoptotic mechanisms start with the release of cytochrome c from mitochondria to induce caspase activation, and consequently, DNA fragmentation. Antioxidant enzymes expressed in response to apoptotic stimulus eradicate the production of ROS. Bcl-2 proteins inhibit the proapoptotic molecules at the mitochondrial level, and chaperones like HSPs and IAPs block caspases, confer resistance and tolerance to damage. In this sense, the first step in limiting cell death is to fully understand the mechanisms involved in apoptotic and survival processes (Figure 4).

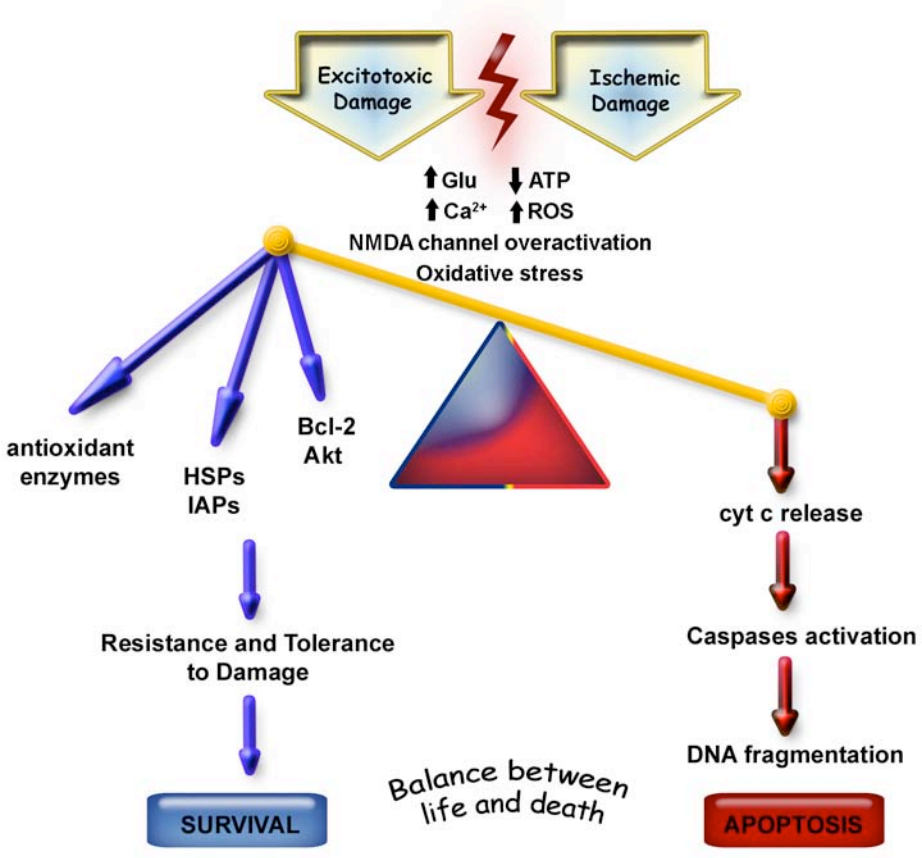


Figure 4. *Schematic representation of the cell death mechanisms induced by excitotoxic/ischemic damage and pro-survival factors responsible for the life and death equilibrium after brain damage.*

1.5. ASTROCYTES IN RESPONSE TO INJURY: REACTIVE ASTROGLIOSIS.

Astrocytes are the predominant glial cell population in the CNS and provide physical support to surrounding neurons and vasculature and give trophic support to neurons by controlling extracellular concentration and homeostasis of several ions and metabolites, including glutamate. A large proportion of the glutamate taken up by astrocytes by their glutamate transporters GLAST and GLT1 (EAAT1 and EAAT2) is converted to glutamine by an enzyme, glutamine synthethase (GS). Exclusively localized in astrocytes, glutamine is then shuttled to neurons, which convert it back to the neurotransmitter glutamate [195, 196, 201, 202]. A depletion of these astroglial transporters raises extracellular glutamate concentrations and increases neuronal damage by excitotoxicity. In the normal CNS, astrocytes are classically divided into three major types according to their morphology and spatial organization: protoplasmic astrocytes in grey matter, fibrous astrocytes in white matter and radial astrocytes surrounding ventricles. Glial fibrillary acidic protein (GFAP) is an intermediate filament protein which is known to be expressed in astrocytes, although its precise contributions to astroglial physiology and function are still not clear. The upregulation of GFAP following injury has been the long-standing pathological observation and the main landmark of reactive astrogliosis in neurodegenerative conditions and acute neuronal damage, including after excitotoxic cell death [203]. In addition, injury-induced changes in intermediate filaments also induce de novo expression of vimentin, which is expressed in the embryonic brain. Early astroglial activation can be seen within hours after injury when the astrocytes generally show an increase of GFAP immunoreactivity associated to cell hypertrophy and sometimes proliferation. The morphological and functional responses of astrocytes may depend on the type of insult [204]. The absence of intermediate filament (IF) proteins in astrocytes leads to attenuation of reactive gliosis with distinct pathophysiological and clinical consequences. Studies with GFAP -/- and Vimentin -/- mice after trauma have proposed that the effect of reactive astrocytes involves two stages: they may first play a beneficial role in the acute stage after CNS injury and later act as inhibitors of CNS regeneration [205]. The absence of astrocyte IFs associates with the diminished ability of astrocytes to respond to hypoosmotic stress. Astroglial cytoskeletal changes are

accompanied by a reactive response that has been shown to have an important role for BBB (blood-brain barrier) repair, reduced brain edema and regulation of blood flow by controlling the blood-CNS interface. Reactive astrocytes are also involved in the regulation of extracellular ions and neurotransmitter levels, as well as the repair of the extracellular matrix (ECM) by secreting ECM proteins. However, whether prolonged reactive astrocytic response is beneficial in neuronal recovery is still controversial. Astrocytes, together with microglial cells, endothelium and infiltrating leukocytes are the major players of the inflammatory response after damage and they have been shown to produce inflammatory cytokines such as IL-1, IL-6 and TNF-α, and the inflammatory enzyme iNOS, a key molecule in oxidative stress [206-210]. In contrast, other factors such as TGF-β and trophic molecules are also produced by astrocytes and may have protective roles [211]. As the damage progresses, a proportion of the surviving astrocytes in the periphery gradually transform into a glial scar that seals off the affected tissue. Nerve degeneration and neuron-astrocyte interaction through gap junction release neurotrophins by astrocytes is essential for neuronal survival (for review [212]). Neuronal survival-promoting factors released from astrocytes are neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), erythropoietin and activity-dependent neuroprotective protein (for review, [212]). Studies in previous decades have largely established that astrogliosis is a key event in the final lesion outcome after several types of acute damage, but very little is known about the susceptibility of these cells to injury and the possible cell death mechanisms contributing to the termination of the glial response.

1.6. NEURONS AND GLIAL CELLS HAVE DISTINCT SUSCEPTIBILITY TO DAMAGE.

Although the primary focus of studies on cell death mechanisms has been on neurons, it is becoming clear from both *in vivo* and *in vitro* studies that glial cells are also sensitive to apoptotic stimuli. The explanation why, after the same apoptotic stimulus it can cause signalling, execution and regulation of the apoptotic pathways in neurons and glial cells is in continuous study. Neurons are described as the most vulnerable cell type to apoptotic damage, followed by oligodendrocytes, astrocytes, endothelial cells, and microglia [213, 214]. In response to oxidative stress, glial cells are more resistant than neurons:

studies in glial cultures show that following exposure to menadione the extent of oxidative damage to mtDNA differed between glial cell types, with astrocytes being the most resistant and oligodendrocytes as well as microglia being more sensitive [215]. Moreover, the capacity of mitochondrial DNA (mtDNA) repair in cultured oligodendrocytes and microglia was significantly less than in cultured astrocytes, correlating well with each cell type susceptibility to apoptosis [215]. Therefore, it is not the quantity of initial damage that determines sensitivity, rather the amount of damage that persists. Thus, the level of DNA damage at a particular time will reflect the rate of DNA damage versus the rate of repair. *In vivo*, examples that implicate oligodendroglial susceptibility to apoptosis are: extensive white matter damage that occurs after an ischemic insult; inflammatory demyelination, the hallmark of multiple sclerosis, and periventricular leukomalacia, and the neuropathology seen in premature birth associated with severe hypoxia. Furthermore, results of studies on the astroglial susceptibility to damage are still controversial. It has been suggested that astroglial subpopulation susceptibility to damage is dependent on their GFAP content [216], and that protoplasmic astrocytes are more susceptible and die more rapidly after ischemic insults than neurons, implicating that glial dysfunctions may have major consequences in the pathogenesis [217]. Cell type-specific vulnerability to different caspases was studied in neurons and astrocytes, where recombinant caspase-3, -6, -7 or -8 were injected into primary neuronal cells. Only caspase-6 was able to induce apoptosis in neurons and only caspase-3 induces apoptosis in astrocytes [218]. However, it is well established that, in injury paradigms, astroglial cells cope with oxidative stress and avoid cell death by increasing amounts of antioxidant and protective chaperone proteins. Antioxidants are present in high concentrations in astrocytes, which protect the surrounding cells containing neurons from oxidative stress-induced cell death. Cu/Zn SOD overexpression is known to provide astrocytes an increased resistance to oxidative damage [219], and it is recognized as one of the main free radical scavengers in ischemia and excitotoxicity [220]. The remarkably high levels of MnSOD, Cu/ZnSOD and total GSH within cell types determine the protection against cell death and the cell depends on repair mechanisms for the removal of the damage to macromolecules such as DNA [215]. Other astroglial protective mechanisms include small HSPs such as HSP27, which is seen in reactive astrocytes [149] and has been shown to protect cells downstream of cytochrome c release. Metallothionein-II (MT-II) is a metal-binding protein, which plays a key role in the detoxification of

heavy metals and free radicals scavenging [221]. In addition, other neuroprotective proteins such as the phosphoprotein enriched in astrocytes (PEA-15) can divert astrocytes from TNFα triggered apoptosis and has been demonstrated in spinal cord reactive astrocytes [222, 223]. In addition, the ratio of pro- and anti-apoptotic members of Bcl-2 family may determine neuron and glia vulnerability to apoptosis; in astrocyte cultures the expression of Bcl-2 protein was greater compared with neurons, and astrocytes also overexpress MAP kinases, phospho-ERK, and decreased Bax [224]. Finally, anti-apoptotic proteins like IAP family have also been demonstrated in reactive astrocytes [127, 131, 225-228]. Therefore, the presence of the above mentioned protective proteins in astrocytes might account for the absence of massive astroglial cell death in CNS injury models by interfering with the apoptotic process.

1.7. ACTIVATION OF APOPTOTIC PATHWAYS IN ASTROCYTES.

The regulation of astrocyte apoptosis is essential in physiological and pathologic processes in the CNS (for review, [212]). Astroglial cells do undergo cell death *in vitro* in a variety of situations, such as calcium overload, oxidative stress, mitochondrial dysfunction, and treatment with apoptotic inducers as staurosporine and ceramide [229] (Table 6). Glutamate exposition induces in astrocytes caspase-3 activation and DNA fragmentation. Oxidative stress by H_2O_2 exposure causes in astrocytes a loss of mitochondrial membrane potentially followed by cytochrome c release, caspase-3-like protease activation, and induction of calpains, and also NF-κB activation. Cultured astrocytes treated with high concentrations of NO donors lead to apoptosis, causing caspase-11 activation, p53, Bax, and DNA fragmentation. *In vivo*, astrocytic apoptosis has been reported in conditions such as traumatic spinal cord injury, traumatic brain injury [230, 231], ischemia/excitotoxicity [232, 233], and neurodegenerative conditions where it has been shown that apoptosis occurs only in a few astrocytes (Table 6). In general, in vivo studies have reported astroglial expression of several proapoptotic caspases, including caspase-3, caspase-7, caspase-8, caspase-9 or caspase-11, and mitochondrial apoptotic proteins after oxidative stress and neurodegenerative diseases (Table 6). Finally, astroglial cells also suffer from ER stress, activation of caspase-12 and upregulation of ER-resident chaperones such as glucose-regulated proteins (GRPs) [183, 234, 235].

Apoptotic Mechanisms in Astrocytes

	Apoptotic Stimulus	Evidence of caspases, apoptotic molecules or features or cell death	References
In vitro	Glutamate	Caspase-3, DNA fragmentation, AP-1, NFkappaB	[236-238]
	H_2O_2-induced Oxidative stress	p53, DNA fragmentation, caspase-3, cytochrome c, Bax, calpains, NFkappaB	[146, 239-244]
	Nitric oxide	p53, Bax, PI3K	[58, 220, 245, 246]
	Irradiation	Bax, caspase-3, DNA fragmentation	[247, 248]
	Ceramide	JNK, Bax	[80, 249]
	Manganese	Caspase-3, iNOS	[250]
	Oxygen and glucose deprivation (OGD)	CHOP, grp78, grp95, p38MAPK, Bax, procaspase-3, caspase-11, DNA fragmentation	[184, 224, 251, 252]
	Lipopolysaccharide (LPS)	Caspase-11, p38MAPK, NO	[253, 254]
	Adenosine	Apoptotic morphology and decreased cell number	[255]
	Menadione	Cytochrome c, caspase-9	[215]
	S-100β	DNA fragmentation, NO	[256]
	Etanol-induced death	GTPase RhoA, caspase-3, Rho kinase, p38MAPK, JNK, COX-2	[257-259]
	Saline solution	DNA fragmentation	[260]
	Phytanic acid	Cytochrome c	[261]
In vivo	Hypoxia-Ischemia	Cytoplasmic vacuoles, PARP-1, caspase-3, cytochrome c, caspase-9, Bax, type-1 plasminogen activator inhibitor (PAI-1), Bad, p53, DNA fragmentation, caspase-8, Bad	[50, 70, 71, 193, 211, 212, 228, 229, 251, 262-283]
	Excitotoxic injury	Caspase-3, caspase-9, DNA fragmentation, PARP	[284, 285]
	Spinal cord injury	Caspase-3, caspase-8, caspase-9	[286-289]
	Traumatic brain injury	Caspase-3, caspase-7, Bid, tBid, caspase-12, caspase-8, caspase-9, DNA fragmentation	[67, 127, 147, 230, 231, 235, 290-296]

Apoptotic and antiapoptotic mechanisms in neurons and glial cells after damage to the immature brain.

Amyotrophic lateral sclerosis	Caspase-3, DNA fragmentation, COX	[297-299]
Diabetes	Caspase-3, DNA fragmentation	[300, 301]
Neurodegenerative Diseases	Caspase-3, cytochrome c, nuclear condensation, DNA fragmentation, Bax, Bcl-x, cytoplasmic vacuoles	[113, 302-315]

- ***Table 6****. Apoptotic pathway proteins in astrocytes in vitro and in vivo.*

1.8. IMMATURE BRAIN INJURY

Perinatal brain injury as a consequence of asphyxia, cerebral ischemia, cerebral hemorrhage, or intrauterine infection is the main contributor to perinatal morbidity and mortality [276]. In addition, a considerable number of infants who survive perinatal hypoxic/ischemic insults will develop cerebral palsy, severe learning disabilities, and other disorders which are major causes of neurological disability throughout life. In spite of this, studies of acute damage to the immature CNS are generally lacking, although several lines of evidence suggest that the immature brain responds in a particular fashion to brain injuries (for review [316]). This occurs, at least in part, since many of the adult gene expression patterns, neural circuit organization, cell differentiation or myelination has not yet been achieved. During nervous system development, apoptotic or programmed cell death (PCD) mechanisms are triggered in the embryonic stage through postnatal age and are physiological processes of nervous cell differentiation and maturation [6, 29, 317]. In the rat, from age E4 to P14 nearly half of the nervous cells die and levels of proapoptotic proteins are increased in relation to the mature brain [276, 316, 318, 319]. Specifically, in regards to the brain´s response to ischemic or excitotoxic damage, age-related differences are based in the different susceptibility to oxidative stress and the maturation of NMDA receptor patterns at early age. Two features have been proposed for the immature brain that renders it especially sensitive to oxidative stress: poor antioxidant capabilities and high concentration of free iron. The increased vulnerability is subsequently explained by an inability to detoxify accumulated H_2O_2 due to a limited capacity of antioxidant enzymes, especially glutathione peroxidase. In the

developing and immature CNS, NMDA receptors have subunit compositions that allow them to open more easily in response to glutamate and to induce a higher calcium influx than mature receptors [320]. These receptor changes are predominantly responsible for the fact that the immature brain is far more excitable and epileptogenic than the adult brain. During development, expression of the regulator NR2 subunits of the NMDA receptor change from a relatively high level of subtype 2B during the first 2 postnatal weeks in the rat, to a predominance of the 2A subunit in the adult. This is a developmentally regulated alteration in the ratio of NR2A: NR2B is reflected in altered receptor properties [321], including increased Ca^{+2} flux. Although levels of NR2A relative to P21 are low in the P7 rat, there is a higher level of basal phosphorylation, which would contribute to the increased excitability of the NMDA receptor supporting normal cerebral development at this stage but also render it more vulnerable to damage (for review [316]). On the other hand, the glial response and inflammatory reaction after immature brain damage also differs considerably from that of the mature CNS. Injuries in the neonatal periods have been shown to produce minimum astrogliosis, and, although scar increases with age, it is commonly established that immature reactive astrocytes are more growth permissive than adult astrocytes. Moreover, during the first two weeks of postnatal life in the rat two different types of microglial cells coexist. In the white matter tracts there are amoeboid microglial cells which appear during prenatal life deriving from monocytes or primitive/fetal macrophages and maintain several macrophage characteristics such as the expression of major histocompatibility complexes (MHC). In the gray matter there are primitive ramified microglial cells, a transition form towards the formation of adult ramified microglial cells found in the mature CNS and derived from the progressive ramification of amoeboid cells. This will ultimately have consequences for the outcome of brain injury to the immature brain.

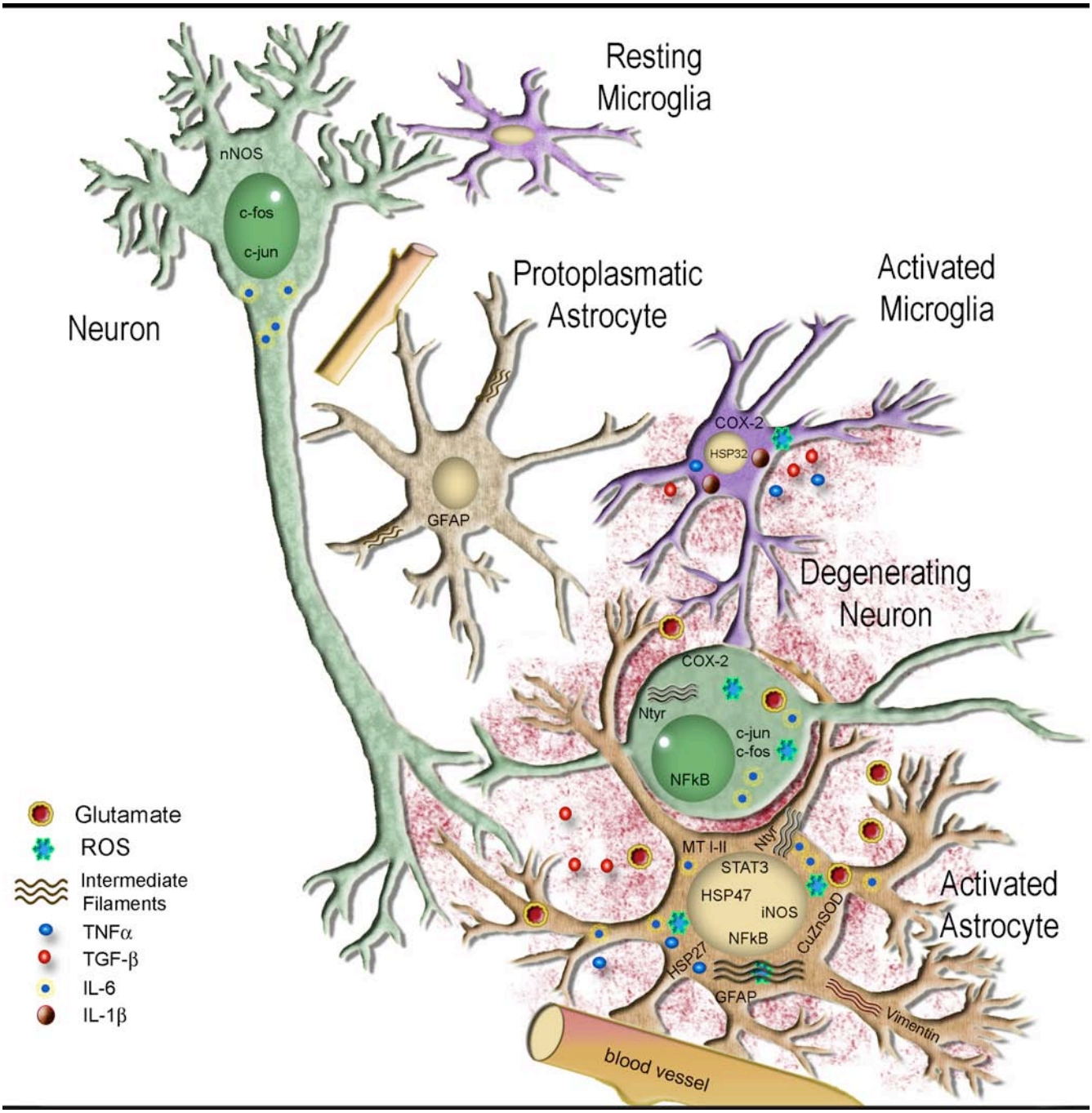


Figure 5*. Schematic representation of blood vessels, astrocytes, neurons and microglial cells after an excitotoxic damage in the immature rat brain. Degenerating neurons present activation of NF-κB and STAT3 and expression of IL-6, and c-fos and c-jun before undergoing cell death. Reactive astrocytes express NF-κB and STAT3 in the nucleus produce the cytokines IL-6, IL-1β, TNFα, and TGF-β1 and induce expression of iNOS. Activated microglia express IL-6 and IL-1β. Protective molecules such as the HSP27 and HSP47 are seen in astrocytes and HSP32 in microglia. Antioxidant enzymes like CuZnSOD and MT-II are expressed also in astrocytes, [149, 209, 210, 322-326].*

In the last decade, our lab has focused on the study of the glial and inflammation response following acute excitotoxicity to the postnatal day 9 rat brain. Neuronal degeneration in the damaged cortex, striatum and hippocampus to a lower extent induce BBB disruption, leukocyte extravasations and glial response. Astroglial activation includes changes in the cytoskeleton and migration, proliferation, and increasing expression of distinct families of genes.

One of the first detectable molecules is the activation of transcription factors Signal transducer and activator of transcription 3 (Stat3) and NFkB in the astroglial nuclei with a few hours after the excitotoxic lesion (Figure 5) [327] and preceding upregulation of GFAP de novo vimentin expression, cell hypertrophy and metabolic changes. Later on, astroglial cells express proinflamatory molecules; IL-1, TNFα, IL-6 and iNOS, but also the anti-inflammatory cytokine TGF-β1, which is maintained until the formation of the glial scar. Microglial cells increase lectin binding and express MHCI [328] and MHCII to a lower extent. In addition, activated microglia express the inflammatory related enzyme COX-2, the pro-inflammatory cytokine IL-1β, and also produce the anti-inflammatory cytokine TGF-β1 and the chaperone HSP32 [209]. As a consequence of oxidative stress, astroglial cells become nitrated and activate caspase-3, but also induce expression of protective mechanisms like the antioxidant Cu/Zn SOD and the metal binding proteins MT-II; and activate stream-activated chaperones like HSP47 and HSP27 [149]. However, little is known about the susceptibility of astrocytes and glial cells in general after acute immature damage and the activation of proapoptotic and antiapoptotic mechanisms within these cells.

2. AIMS

General aim and Hypothesis

The general purpose of this thesis was to study the apoptotic and anti-apoptotic mechanisms in neurons and glial cells after acute excitotoxic damage to the immature rat brain. Glial cells and, in particular, astrocytes show more resistance to death than neurons after damage. Our hypothesis is that glial cells have a determined pattern in the expression of molecules that award resistance in the balance between survival and death.

Specific aims

I. To analyze the spatial-temporal pattern of caspase-3 activation in neurons and glial cells.

II. To determine the rate and temporal progression of neuronal and glial apoptotic cell death in the damaged brain.

III. To study the spatial-temporal activation of the intrinsic and extrinsic apoptosis pathways in neurons and glial cells.

IV. To evaluate possible alternative non-apoptotic roles of caspase-3 in astroglial cells.

V. To evaluate several IAPs as candidates for caspase-3 inhibition in astrocytes.

VI. To evaluate whether Heat Shock Proteins maybe involved in caspase-3 inhibition in astrocytes.

3. ARTICLES

ARTICLE I

"*Caspase-3 activation in astrocytes following postnatal excitotoxic damage correlates with cytoskeletal remodeling but not with cell death or proliferation*"

GLIA 55:954 - 965 (2007).

Caspase-3 Activation in Astrocytes Following Postnatal Excitotoxic Damage Correlates With Cytoskeletal Remodeling but not With Cell Death or Proliferation

ABSTRACT

Caspase-3 has classically been defined as the main executioner of programmed cell death. However, recent data supports the participation of this protease in non-apoptotic cellular events including cell proliferation, cell cycle regulation, and cellular differentiation. In this study, astroglial cleavage of caspase-3 was analyzed following excitotoxic damage in postnatal rats to determine if its presence is associated with apoptotic cell death, cell proliferation, or cytoskeletal remodeling. A well-characterized in vivo model of excitotoxicity was studied, where damage was induced by intracortical injection of N-methyl-D-asparate (NMDA) in postnatal day 9 rats. Our results demonstrate that cleaved caspase-3 was mainly observed in the nucleus of activated astrocytes in the lesioned hemisphere as early as 4 h post-lesion and persisted until the glial scar was formed at 7 – 14 days, and it was not associated with TUNEL labeling. Caspase-3 enzymatic activity was detected at 10 h and 1 day postlesion in astrocytes, and co-localized with caspase-cleaved fragments of glial fibrillary acidic protein (CCP-GFAP). However, at longer survival times, when astroglial hypertrophy was observed, astroglial caspase-3 did not generally correlate with GFAP cleavage, but instead was associated with de novo expression of vimentin. Moreover, astroglial caspase-3 cleavage was not associated with BrdU incorporation. These results provide further evidence for a nontraditional role of caspases in cellular function that is independent of cell death and suggest that caspase activation is important for astroglial cytoskeleton remodeling following cellular injury.

GLIA 55:954–965 (2007)

Caspase-3 Activation in Astrocytes Following Postnatal Excitotoxic Damage Correlates With Cytoskeletal Remodeling but not With Cell Death or Proliferation

LAIA ACARIN,[1]* SONIA VILLAPOL,[1] MARYAM FAIZ,[1] TROY T. ROHN,[2] BERNARDO CASTELLANO,[1] AND BERTA GONZÁLEZ[1]
[1]*Medical Histology, Department of Cell Biology, Physiology and Immunology, Faculty of Medicine and Institute of Neurosciences, Autonomous University of Barcelona, Spain*
[2]*Department of Biology, Boise State University, Boise, Idaho*

KEY WORDS
immature; astrogliosis; cytoskeleton; caspase

ABSTRACT
Caspase-3 has classically been defined as the main executioner of programmed cell death. However, recent data supports the participation of this protease in non-apoptotic cellular events including cell proliferation, cell cycle regulation, and cellular differentiation. In this study, astroglial cleavage of caspase-3 was analyzed following excitotoxic damage in postnatal rats to determine if its presence is associated with apoptotic cell death, cell proliferation, or cytoskeletal remodeling. A well-characterized *in vivo* model of excitotoxicity was studied, where damage was induced by intracortical injection of *N*-methyl-D-asparate (NMDA) in postnatal day 9 rats. Our results demonstrate that cleaved caspase-3 was mainly observed in the nucleus of activated astrocytes in the lesioned hemisphere as early as 4 h postlesion and persisted until the glial scar was formed at 7–14 days, and it was not associated with TUNEL labeling. Caspase-3 enzymatic activity was detected at 10 h and 1 day postlesion in astrocytes, and co-localized with caspase-cleaved fragments of glial fibrillary acidic protein (CCP-GFAP). However, at longer survival times, when astroglial hypertrophy was observed, astroglial caspase-3 did not generally correlate with GFAP cleavage, but instead was associated with *de novo* expression of vimentin. Moreover, astroglial caspase-3 cleavage was not associated with BrdU incorporation. These results provide further evidence for a nontraditional role of caspases in cellular function that is independent of cell death and suggest that caspase activation is important for astroglial cytoskeleton remodeling following cellular injury.

INTRODUCTION

Caspase-3 is considered one of the major executioners of apoptosis and has classically been viewed as a terminal event in the process of programmed cell death. Caspase-3 is proteolytically activated into two catalytic subunits of 10 kDa (p10) and 20 kDa (p20), which can cleave numerous intracellular substrates including cytoskeletal elements, degrading proteases, and nuclear enzymes involved in DNA repair (Marks and Berg, 1999; Springer et al., 2001) to name just a few. Accordingly, caspase-3 activation has been described in neuronal cells following specific types of central nervous system (CNS) insults including traumatic brain injury and ischemic/excitotoxic damage (Beer et al., 2000; Brecht et al., 2001; Chen et al., 1998; Manabat et al., 2003; Namura et al., 1998; Nath et al., 2000; Pulera et al., 1998). In all of these conditions, the participation of executioner caspase activation and subsequent apoptosis in the neuronal cell death process has largely been established both in the adult and postnatal brain. Similarly, *in vitro* studies have also suggested that caspase-3 proteolytic activity plays a crucial role in excitotoxin-induced neuronal apoptosis (Allen et al., 1999; Du et al., 1997; Tenneti and Lipton, 2000), although caspase-3 activation and DNA fragmentation often do not co-localize within the same cell at the same time (Brecht et al., 2001). In addition, expression of caspase-3 has been described in oligodendrocytes (Beer et al., 2000; Nottingham and Springer, 2003) and astrocytes following CNS damage (Beer et al., 2000; Benjelloun et al., 2003; Mouser et al., 2006; Narkilahti et al., 2003; Su et al., 2000).

Recent studies, however, provide substantial evidence for caspase function in non-apoptotic cellular events such as cell cycle regulation, migration, and differentiation in a variety of cell types (McLaughlin, 2004; Schwerk and Schulze-Osthoff, 2003). Although the preponderance of data has emerged from work in the immune system and peripheral organs, several studies have also suggested non-apoptotic roles of caspase-3 in the CNS. Under physiological conditions, caspase-3 has been implicated in neuronal cytoskeletal changes (Rohn et al., 2004), in synaptic remodeling (Dash et al., 2000; Shimohama et al., 2001), in neuronal survival associated with preconditioning (Garnier et al. 2003; McLaughlin et al., 2003; Tanaka et al., 2004), in the differentiation of cerebellar Bergmann glial cells (Oomman et al., 2004, 2005, 2006), and as a marker of astroglial subpopulations

Grant sponsor: Ministry of Education and Science; Grant number: BFU2005-02783, 2005SGR-00956.

*Correspondence to: Laia Acarin, Medical Histology, Torre M5, Faculty of Medicine, Universitat Autonoma Barcelona, Bellaterra 08193 (Barcelona), Spain. E-mail: Laia.acarin@uab.es

Received 22 December 2006; Accepted 10 April 2007

DOI 10.1002/glia.20518

Published online 8 May 2007 in Wiley InterScience (www.interscience.wiley.com).

(Noyan-Ashraf et al., 2005). Accordingly, following excitotoxic damage in the neonatal brain we have demonstrated that presence of cleaved caspase-3 in nitrated reactive astrocytes in the absence of cell death (Acarin et al., 2005). Nevertheless, alternative roles for caspase-3 in CNS astrocytes after injury have not been described.

The first aim of the present study was to describe the pattern, time course, and cellular distribution of cleaved caspase-3 and TUNEL labeling after postnatal excitotoxicity by using a well characterized *in vivo* lesion model in the immature rat brain (Acarin et al., 1999a,b, 2002, 2005). Brain damage as a consequence of perinatal cerebral hypoxia/ischemia and stroke is a major cause of acute mortality and severe chronic disabilities, and excitotoxicity is one of the crucial underlying mechanisms. Several evidences suggest that in comparison to the adult brain, the immature brain responds in a particular fashion to brain injuries, partly due to the fact that many of adult gene expression patterns, neural circuits organization, cell differentiation, and myelination have not yet been completed (Ferriero, 2004; Vannucci and Hagberg, 2004). Furthermore, as astroglial cells were the main cell type showing cleaved caspase-3 but not apoptotic cell death after neonatal excitotoxicity, the second aim was to elucidate the putative role that caspase-3 may play in proliferation and cytoskeletal reorganization in reactive astrocytes. Our findings suggest a participation of this protease in astroglial remodeling of intermediate filaments containing glial fibrillary acidic protein (GFAP) and vimentin after damage, more so than a contribution to cell death or modulation of proliferation.

MATERIALS AND METHODS

Excitotoxic Lesions

Nine-day-old Long-Evans black-hooded rat pups of both sexes were placed in a stereotaxic frame adapted for newborns (Kopf) under isofluorane anaesthesia. The skull was opened using a surgical blade, and 0.15 μL of saline solution (0.9% NaCl, pH 7.4) containing 20 nmols of *N*-methyl-D-aspartate (NMDA) (Sigma, M-3262, Germany) were injected into the right sensorimotor cortex. Control animals received an injection of 0.15 μL of the vehicle saline solution. After suture, pups were placed in a thermal pad and maintained at normothermia before being returned to their mothers. Experimental animal work was conducted according to Spanish regulations, in agreement with European Union directives. All experimental procedures were approved by the ethical commission of the Autonomous University of Barcelona. All efforts were made to minimize animal suffering.

Survival Times and Sample Processing

Rats were sacrificed at 4 and 10 h and 1, 3, 5, 7, and 14 days after NMDA or saline injection. For histological procedures, rats were anesthetized by ether inhalation and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were post-fixed in the same fixative for 2 h and sunk in a 30% sucrose solution before being frozen with dry CO_2. Coronal sections (30-μm-thick) were obtained using a Leitz cryostat. For caspase-3 activity, rats were sacrificed by decapitation, brains were quickly removed and the cortices dissected out, frozen in liquid nitrogen and kept at −80°C. A minimum of four NMDA-injected animals, two saline-injected controls, and two intact controls were used for each survival time and each procedure.

5′-Bromodeoxyuridine Administration

In another set of animals, the thymidine analogue 5′-bromodeoxyuridine (BrdU, Sigma Chemical, St Louis, MO) that incorporates into the DNA of dividing cells during S-phase, was used to label actively proliferating cells. NMDA or saline injected animals were administered intraperitonealy with BrdU (50 mg/kg) diluted in 0.05 M Tris base (TB, pH 7.4) every 2 h for 10 h before sacrifice at 1, 3, 5, and 7 days after NMDA or saline injection.

Immunohistochemistry and Histochemistry

After rinsing for 1 h in 0.05 M tris-buffered saline (TBS), endogenous peroxidase was blocked with 2% H_2O_2 in 70% methanol for 10 min. Sections were rinsed in TBS and TBS + 1% triton X-100 and incubated in blocking buffer (BB, TBS containing 10% FCS, and 1% triton X-100) for 30 min. The sections were then incubated overnight at 4°C and 1 h at room temperature with either primary rabbit anti-cleaved caspase-3 (recognizing the p17/19 kDa fragment, 1:200, Cell Signaling Technology, 9661) or primary rabbit anti-cleaved caspase-3 (recognizing the p17 subunit, 1:600, R&D Systems, AF835) in BB. After washing, sections were incubated for 1 h at room temperature with a biotin-conjugated anti-rabbit secondary antibody (1:200, RPN1004, Amersham Pharmacia), rinsed in TBS + 1% triton and incubated for 1 h at room temperature with HRP-conjugated streptavidin (1:400, PO364, Dakopatts). Finally, the peroxidase reaction product was visualized by incubating the sections in 100 mL of TB containing 50 mg 3,3′diaminobenzidine (DAB) and 33 μL hydrogen peroxide. As negative controls for immunohistochemistry, sections were incubated in media lacking primary antibody.

Double staining procedures were used for the simultaneous visualization of cleaved caspase-3 and NeuN as a neuronal marker; GFAP and vimentin as astroglial markers; Adenomatus Polyposis Coli (APC) as a marker for oligodendrocytes, GFAP-CCP as a marker of caspase-cleaved GFAP, BrdU as a marker for proliferating cells, and tomato lectin histochemistry to label microglial cells. For double fluorescent labeling, sections were processed for cleaved caspase-3 labeling as described

earlier but using a Cy3-conjugated anti-rabbit secondary antibody (1:1,000; PA4300V, Amersham Pharmacia). Afterwards, sections were incubated overnight at 4°C and 1 h at room temperature in either monoclonal mouse anti-NeuN (1:1,000; MAB377, Chemicon); polyclonal rabbit anti-GFAP (1:1,800; Z-0224, Dakopatts); monoclonal mouse anti-vimentin (1:1,000; M0725, Dakopatts); monoclonal mouse anti-APC (CC-1) (1:500, OP80, Calbiochem); monoclonal mouse anti-BrdU (1:80, B5002, Dakopatts); or polyclonal rabbit anti-CCP-GFAP (Mouser et al., 2006) diluted in BB. Sections were then washed in TBS + 1% triton X-100 and incubated for 1 h at room temperature with either Cy2-conjugated anti-rabbit secondary antibody (1:1,000; PA-42004, Amersham Pharmacia) or Cy2-conjugated anti-mouse secondary antibody (1:1,000; PA42002, Amersham Pharmacia). For tomato lectin histochemistry, sections were incubated for 2 h at 37° in tomato lectin (1:150, L0651, Sigma) in TBS + 1% triton X-100. After washing, sections were incubated for 1 h at room temperature with Cy2-conjugated avidin (1:1,000; PA-42000, Amersham Pharmacia). Selected mounted sections were stained with a fluorescent nuclear marker by incubating slides for 10 min in a DAPI (D9542, Sigma) solution at a concentration of 0.00125 μg/mL. After washing, sections were dehydrated, air-dried, and cover-slipped using DPX. Fluorescence and light microscopy digital images were captured with a Nikon Digital Eclipse DXM1200 color camera attached to a Nikon E-800 microscope. Confocal images were obtained using a LEICA TCS SP2 AOBS confocal microscope.

Terminal dUTP Nick End Labeling (TUNEL) Staining and Double Labeling with Cleaved Caspase-3, and Neuronal and Glial Markers

For TUNEL staining, tissue sections were rinsed in Tris buffer (10 mM, pH 8) and EDTA (5 mM) and then incubated in the same buffer plus Proteinase K (20 μg/mL) for 15 min at room temperature. After several washes with EDTA (5 mM), sections were incubated for 10 min in TdT buffer (Tris 30 mM, 140 mM sodium cacodilate, 1 mM cobalt chloride, pH 7,7). Sections were then incubated in TdT buffer plus 0.161 U/μL TdT enzyme (Terminal Transferase, 3333566 Roche, Manheim, Germany) and 0.0161 nmol/μL of biotin-16-dUTP (1093070, Roche, Manheim, Germany) for 30 min at 37°C. The reaction was stopped by washing the sections in citrate buffer (300 mM sodium chloride, 30 mM sodium citrate, 5 mM EDTA). After several washes with TBS, sections were incubated with HRP-conjugated streptavidin (1:400, SA5004, Vector Laboratories) and the peroxidase reaction product was visualized in a solution containing 0.02% DAB, 2.4% nickel ammonium, 0.04% chloride ammonium, 0.2% glucose D+, and 0.0027% glucose oxidase in 0.1 M acetate buffer (pH 6.0).

For double labeling, TUNEL-stained sections were incubated with either anti-NeuN, anti-GFAP, anti-APC, or tomato lectin as described in the previous section but using biotin-conjugated secondary antibodies: biotin-conjugated anti-rabbit secondary antibody (1:200, RPN1004, Amersham) for GFAP or biotin-conjugated anti-mouse secondary antibody (1:200, RPN1001, Amersham) for NeuN and APC and incubated for 1 h at room temperature with HRP-conjugated streptavidin (1:400, PO364 Dakopatts). Sections incubated with tomato lectin were directly incubated in the HRP-conjugated streptavidin (1:400, PO364 Dakopatts). Finally, peroxidase reaction product was visualized by incubating the slides in 100 mL of tris buffer containing 50 mg DAB and 33 μL of hydrogen peroxide.

For TUNEL fluorescent labeling and double immunostaining with cleaved caspase-3, free-floating sections were first incubated with the primary antibody anti-cleaved caspase-3 (1:1,000; Cell Signaling Technology, 9661), which was visualized by using Cy3-conjugated anti-rabbit secondary antibody (1:1,000; PA-42004, Amersham). Afterwards, sections were mounted on slides and TUNEL labeling was performed as described above but using Cy2-conjugated streptavidin (1:1,000; PA-42000, Amersham Pharmacia) to visualize the staining.

Caspase-3 Activity Assay

Ipsilateral cortices of intact controls, saline-injected, and NMDA-injected animals, were weighed and homogenized in ice-cold Tris/HCl buffer containing a cocktail of protease inhibitors. Homogenates were centrifuged (12,000 rpm) for 10 min at 4°C and supernatants were extracted and assayed for caspase-3 activity using EnzCheck assay kit #1 containing Z-DEVD-AMC substrate (E-13183 Molecular Probes, Invitrogene), following Manufacturer's instructions. Caspase activity was measured in 50 μL of sample for 30 min using a fluorimeter at 340/360 nm of excitation and 440/460 nm emission, using appropriate filters. Arbitrary fluorescent units were converted into micromoles of AMC release using a standard curve and standardized to total protein in each sample. Enzyme activity is shown as micromolar AMC released/mg total protein. As a control, the protease inhibitor Ac-DVED-CHO was incubated with alternate samples and controls to inhibit caspase-3-like activity.

Cell Number Quantification

Digital images were captured at different magnifications with a Nikon Digital Eclipse DXM1200 color camera attached to a Nikon E-800 microscope using the software ACT-1 2.20 (Nikon Corporation). TUNEL-positive cells, cleaved caspase-3-positive cells, and cleaved caspase-3/GFAP double positive cells were counted in 20× micrographs from the cortical lesion core. For the analysis of TUNEL-GFAP double-labeled cells, TUNEL-positive and double TUNEL/GFAP-positive cells of the same area were counted in micrographs taken at 40× from the cortical lesion core. In all cell counts, at least 10 consecutive sections were counted for each animal and averaged.

A minimum of three animals for each survival time were used.

Data Processing and Statistical Analysis

All results are expressed as mean ± standard error mean (SEM). Statistical analysis was performed using StatView software, where differences were evaluated by one-way analysis of variance (ANOVA) followed by Fisher's PLSD *post-hoc* test comparisons to determine significant differences ($P < 0.05$) between survival times and cell types.

RESULTS

Injection of the excitotoxin NMDA into the right sensorimotor cortex of postnatal day 9 rat pups caused a lesion involving neuronal loss and a glial response in the entire thickness of the cortex and the dorsal striatum at the level of the injection site, which has been previously described in detail (Acarin et al., 1996; 1999a,b, 2000a). Injection of a control saline solution resulted in slight tissue disruption restricted to the area of the needle track and a focal and transient glial response that lasted until three days postinjection.

Distribution and Time Course of Cleaved Caspase-3 Immunoreactivity and Caspase-3 Enzymatic Activity

Cleaved caspase-3 immunoreactivity in both intact control and saline-injected brains, was seen in the ventricle walls and meninges and in cells accumulated in the cingulum of the corpus callosum (Fig. 1B). In contrast, in NMDA-injected animals, cleaved caspase-3-positive cells were found in the ipsilateral hemisphere at all survival times examined. Both primary antibodies used for the detection of cleaved caspase-3 (see methods section) showed similar results. Cleaved caspase-3 labeling was commonly found in the nuclei, and in a minority of cells in the perinuclear region (Fig. 1). At 4 h postlesion, scattered cleaved caspase-3-positive cells were present in the damaged cortex (Figs. 1A,C), mainly in the periphery of the degenerating area. At 10 h and 1 day postlesion, extensive dark cleaved caspase-3 staining was seen throughout the degenerating cortical area, namely in the upper cortical layers and the medial cortex, and in the ipsilateral dorsal striatum (Figs. 1D–F). The maximum number of cleaved caspase-3-positive cells in the neurodegenerating cortex was seen at day 1 (Fig. 2), when fainter caspase staining was also observed in adjacent cortex and the corpus callosum. At days 3 and 5, cleaved caspase-3 staining was still evident in the cortical neurodegenerative area (Figs. 1G–I) but the number of positive cells was slightly diminished (Fig. 2). In addition, cleaved caspase-3-positive cells were also present in the dorsal striatum, and in the deeper cortical layer VI even at caudal levels far from the lesioned area (Fig. 1G). From day 7, the number of positive cells was clearly reduced and cleaved caspase-3 immunoreactivity became restricted to the glial scar, with darker staining observed in the upper cortical layers and fainter staining in the lower cortical layers and corpus callosum (Figs. 1J–L and 2).

The analysis of caspase-3 activity using a fluorimetric assay showed no differences within the different postnatal ages, both in intact controls and in saline-injected controls. However, NMDA-injected cortices showed significant enzymatic caspase-3 activity at 10 h and 1 day postlesion, but this activity was not significantly different from controls at shorter or longer survival times (Fig. 3).

Identification of Cleaved Caspase-3 Positive Cells by Double Labeling with Specific Cellular Markers

In control animals and in the contralateral hemispheres of lesioned animals, cleaved caspase-3-positive cells were identified as GFAP-positive astroglial cells in the cingulum of the corpus callosum (data not shown). In the damaged hemisphere of lesioned animals, cleaved caspase-3 was seen both in neurons and astrocytes (see Fig. 4). Mainly at 10 h and 1 day postlesion, cleaved caspase-3 immunolabeling was observed in NeuN-positive neuronal cells within the degenerating core and in the proximal lesion border (Figs. 4A,B), but not in the distant caudal cortex (Fig. 4C). Cleaved caspase-3 labeling in neurons was both nuclear and cytoplasmic (Figs. 1E,F and 4B).

Colocalization of cleaved caspase-3 in the nuclei of astroglial cells was evident in the damaged cortex and the adjacent corpus callosum from 4 h postlesion, the first survival time analyzed (Figs. 4D and 5). At 10 h postlesion, when a decrease in GFAP immunoreactivity is observed in the lesion core (Acarin et al., 1999b), double-labeled cleaved caspase-3/GFAP-positive astrocytes comprised 35% of the total number of cleaved caspase-3-positive cells, the lowest percentage seen at all time points examined (see Fig. 5). At day 1 postlesion, the presence of cleaved caspase-3 was associated with astroglial hypertrophy and increased GFAP labeling (Figs. 4E,F) and by the onset of vimentin expression (Acarin et al., 1999b). Astrocytes in cortical layer VI and in the striatum showed stronger cleaved caspase-3 immunoreactivity than those located in the corpus callosum. From 3 days postlesion, astrocytes were the main population showing cleaved caspase-3 immunoreactivity (see Fig. 5). At this time and at 5 days postlesion, increased GFAP immunoreactivity and astroglial hypertrophy was observed throughout the neurodegenerative area. The observed astroglial hypertrophy was also associated with vimentin immunoreactivity as well as the presence of cleaved caspase-3 in the nucleus (Figs. 4G–H). From 7 days postlesion, reactive hypertrophied astrocytes located in the glial scar showed cleaved caspase-3 immunoreactivity in the nucleus and strong GFAP (Fig. 4I) and vimentin labeling.

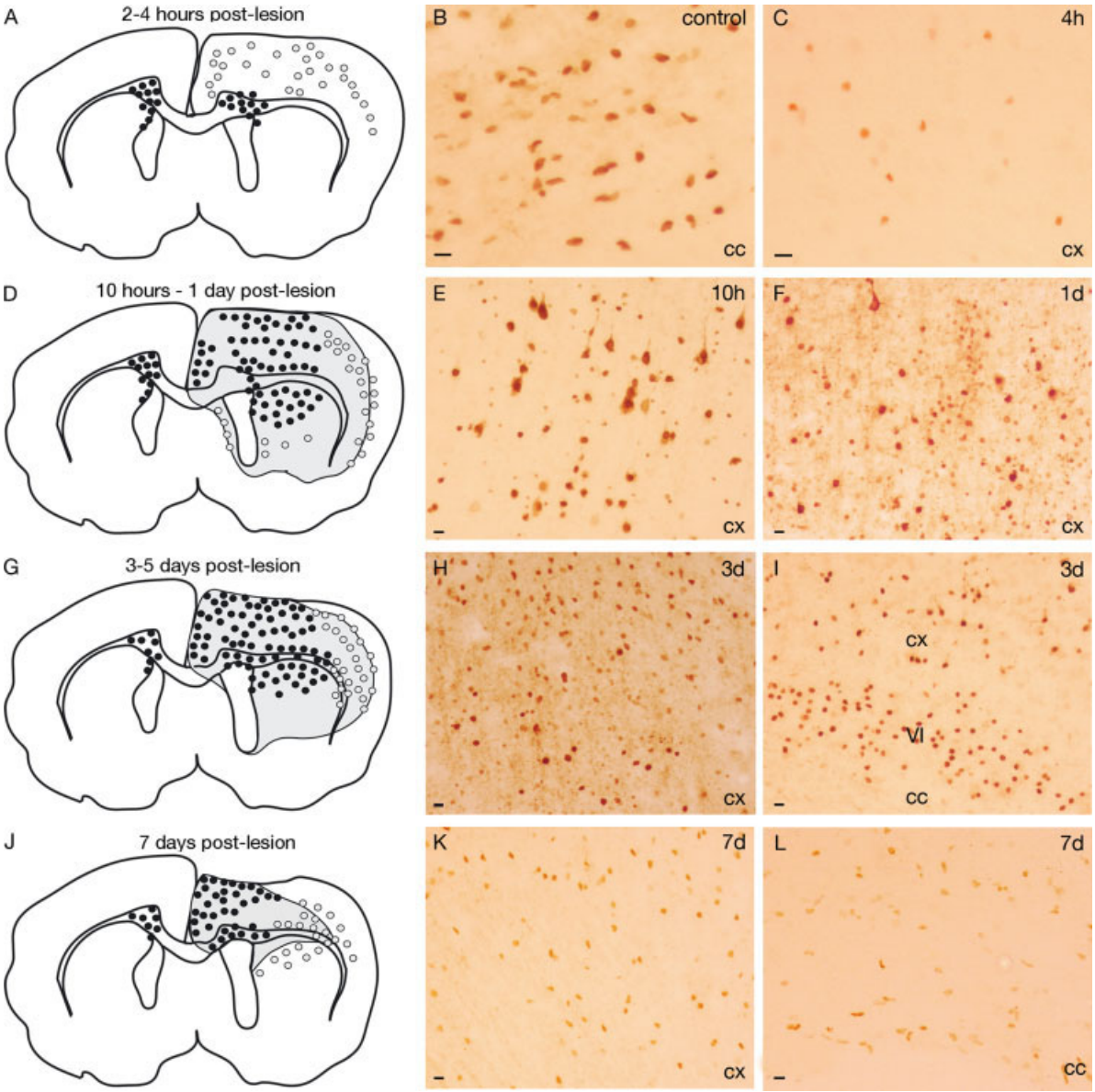


Fig. 1. Temporal and spatial distribution of cleaved caspase-3 immunoreactive cells at different survival times following a cortical excitotoxic lesion in the immature brain. Lesioned area is encircled and shown in light grey in the ipsilateral right hemisphere from 10 h post-lesion (**D**, **G**, **J**). Cells strongly labeled for cleaved caspase-3 are shown in black circles, whereas mildly positive cells are depicted in white circles. In the contralateral control hemisphere cleaved caspase-3 cells are found in the cingulum of the corpus callsoum (left hemisphere in **A**, **B**). In NMDA-injected ipsilateral hemisphere, immunoreactive cells are seen in the cortex and corpus callosum at all survival times (**A**–**L**). cc, corpus callosum; cx, cortex; VI, cortical layer VI. Scale bars = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

We did not find, at any survival time, colocalization between cleaved caspase-3 immunoreactivity and tomato lectin, a specific marker for either microglia/macrophages or endothelial cells (Fig. 4J). In addition, colocalization was not evident between cleaved caspase-3 and the APC-positive oligodendrocytes (Figs. 4K–L).

Association Between Cleaved Caspase-3 and Proliferation

To assess the role of caspase-3 in cellular proliferation, the cleavage of caspase-3 was examined in BrdU positive cells in animals injected with the thymidine analogue prior to sacrifice. Few BrdU-positive cells showed cleaved caspase-3 labeling in the nucleus, while the majority of cleaved caspase-3 labeled nuclei did not show BrdU incorporation at any survival times studied (Figs. 6A,B).

Association Between Cleaved Caspase-3 and the Presence of Caspase-Cleaved GFAP Fragments

Presence of cleaved caspase-3 in astrocytes was associated with the presence of caspase-cleaved GFAP fila-

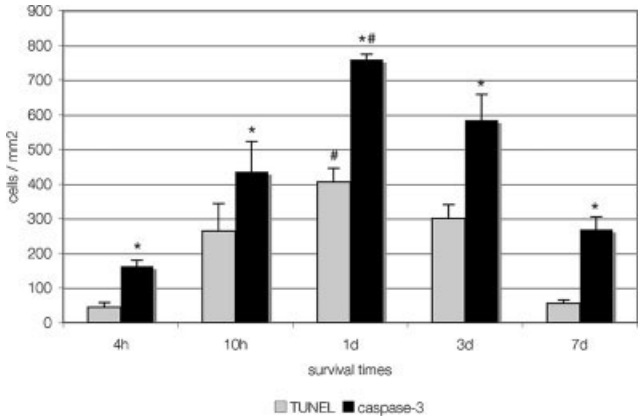


Fig. 2. Quantification of cleaved caspase-3 positive cells and TUNEL positive cells in the excitotoxically injured cortex of postnatal rats at different survival times ($n = 4$ for each labeling and survival time). Cleaved caspase-3-positive cells outnumber TUNEL-positive cells at all survival times ($^*P < 0.05$). Maximum density of both cleaved caspase-3-positive cells and TUNEL-positive cells is seen at day 1 postlesion ($^{\#}P < 0.05$).

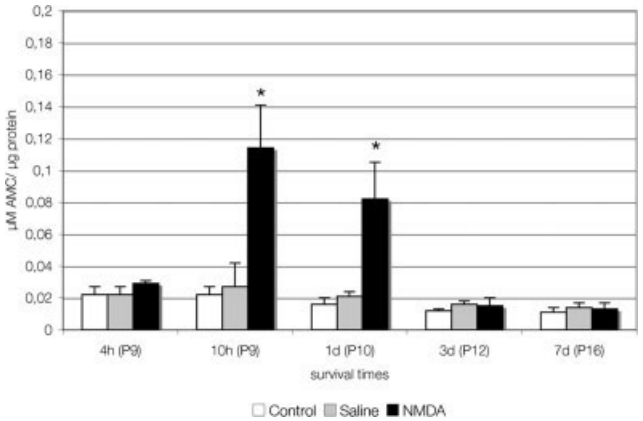


Fig. 3. Caspase-3 enzymatic activity in the cortex of intact control postnatal rats of 9 days of age (P9), P10, P12, P14, and P16 ($n = 2$ for each age); and at different survival times following saline ($n = 2$ for each survival time) or NMDA-injection ($n = 4$ for each survival time). Caspase-3 enzymatic activity is significant at 10 h and 1 day postlesion in NMDA-injected cortex ($^*P < 0.05$) in comparison to both control and lesioned cortices at 4 h, 3 or 7 days postlesion, when caspase-3 activity was not different from controls.

ments by using a recently described site-directed caspase-cleavage antibody specific to GFAP, termed CCP-GFAP (Mouser et al., 2006). CCP-GFAP immunostaining was located within the lesion core from 10 h until the last survival time examined, peaking at day 1. At 10 h and 1 day postlesion, CCP-GFAP was observed as rounded beads in the cytoplasm of astrocytes, which displayed cleaved caspase-3 in their nuclei (Figs. 6E–H) and were located in the lesion core and immediately surrounding cortex (Fig. 6E). Interestingly, GFAP immunoreactivity was markedly reduced in those cell projections showing CCP-GFAP immunolabeling (Figs. 6F–G). At day 3 postlesion, only scattered reactive astrocytes showed CCP-GFAP within the GFAP-positive filaments (Fig. 6H), and were mainly located in the medial cortex and the upper cortical layers. No association at all was found at 7 days between CCP-GFAP and GFAP (Fig. 6I), and instead CCP-GFAP-positive structures were located within lectin-labeled macrophages (data not shown).

Association Between Cleaved Caspase-3 and TUNEL Labeling

Saline-injection in controls resulted in only a few number of TUNEL-positive cells, being located primarily in the region of the needle track, where tissue disruption occurs, and occasionally in the meninges. In NMDA-injected animals, TUNEL-positive nuclei displaying condensed chromatin, pyknotic nuclei, or apoptotic bodies were seen from 10 h until 7 days postlesion. Quantitative analysis revealed a maximum number of TUNEL-positive cells at 1 day postlesion and decreasing thereafter (see Fig. 2). TUNEL-positive apoptotic cells were located in the cortical lesion site but also extended to the dorsal striatum, the septum, and CA fields of the rostral hippocampus, but were rarely found in the corpus callosum. In general, cleaved caspase-3-positive nuclei always outnumbered those showing TUNEL-labeling at all times examined (see Fig. 2). Although some cells showed both cleaved caspase-3 and TUNEL staining, many cleaved caspase-3-positive cells did not colocalize with TUNEL and furthermore, many TUNEL-positive cells did not display cleaved caspase-3 labeling (Figs. 6C,D).

Double Labeling for TUNEL and Specific Cell Markers

Qualitatively, we could observe that most TUNEL-positive cells observed at all time points postlesion were identified as NeuN-positive neuronal cells (Figs. 7A,B) and that TUNEL/NeuN double labeled cells were more frequent than cleaved caspase-3/NeuN double positive cells (compare with Figs. 4A–C). TUNEL-positive neurons often showed nuclear fragmentation (Fig. 7A) and were located in the cortical degenerating area (Figs. 7A,B), cortical layer VI, the dorsal striatum, and the rostral hippocampus CA field (data not shown).

In addition, although most lectin-positive microglial cells did not show TUNEL labeling (Fig. 7C), some microglia/macrophages with characteristic round or ameboid phagocytic forms at 5 days postlesion were found (Fig. 7D).

Analysis of TUNEL/GFAP double-labeled sections showed few astrocytes with TUNEL-positive nuclei, contrasting with the high presence of cleaved caspase-3 in reactive astrocytes. At 10 h, when massive TUNEL staining was observed in neuronal cells, only 8% of all TUNEL-positive cells were astrocytes (Fig. 5). These cells were found close to the tissue disrupted at the site of the needle track, and in the corpus callosum (Figs. 7E–F). At days 3–5, although astrocytes did not gener-

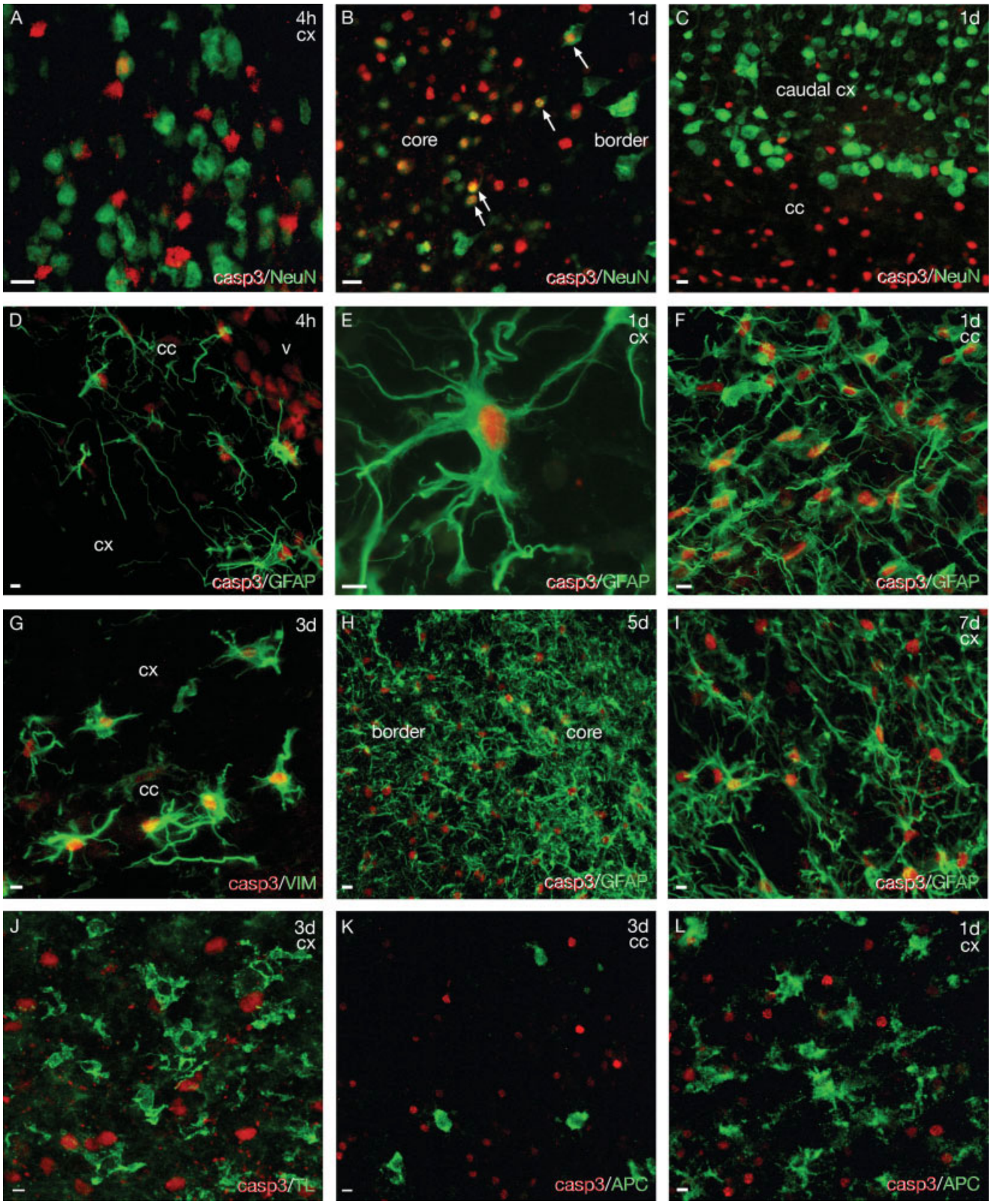


Fig. 4. Identification of cleaved caspase-3-positive cells at different survival times following a cortical excitotoxic lesion in the postnatal brain. Caspase-3 (casp3) labeling is shown in red, whereas the neuronal marker NeuN (in **A**–**C**), the astroglial markers GFAP (in **D**–**F**, **H**, and **I**) and vimentin (VIM) (in **G**), the microglial marker tomato lectin (TL) (in **J**), and the oligodendroglial marker APC (in **K** and **L**) are shown in green. NeuN-positive neuronal cells do not generally show cleaved caspase-3 labeling at 4 h postlesion (A), but labeling is seen in neurons at 10 h and 1 day postlesion (arrows in B) in the lesion core and the proximal border (B) but not in the distal caudal cortex (c). In contrast, astrocytes show nuclear cleaved caspase-3 as early as 4 h postlesion (D) and persists until the last survival time analyzed (I). Cleaved caspase-3 positive astrocytes are seen in the corpus callosum (cc) (F), the lesion core and cortical border (H) and in the cortical glial scar (I). Cleaved caspase-3 colocalizes with vimentin-positive astrocytes (G). In contrast, no TL-positive cells (J) or APC-positive oligodendrocyte (K, L) show cleaved caspase-3 labeling. cc, corpus callosum; cx, cortex; v, ventricle. Scale bars = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

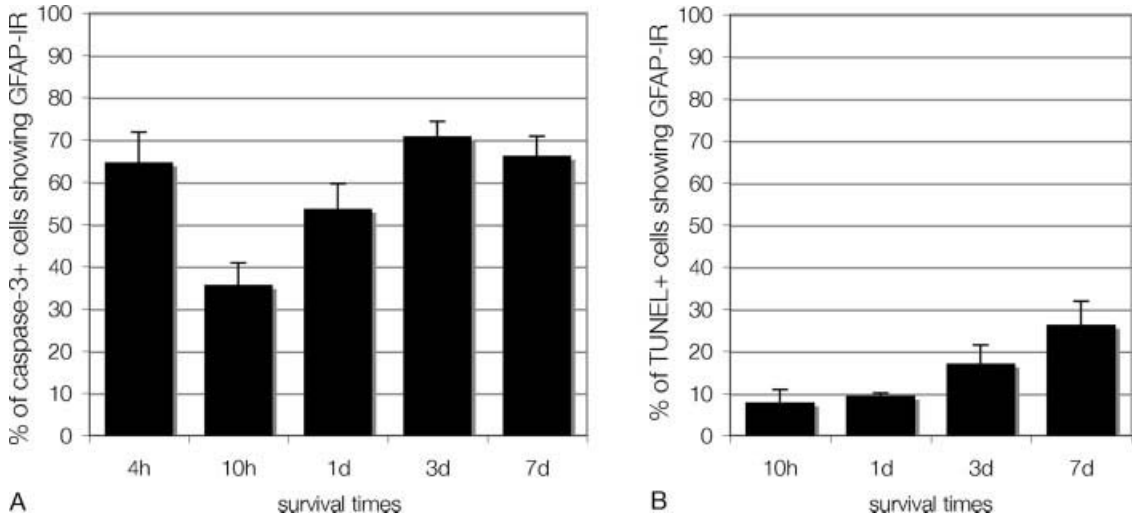


Fig. 5. Quantification of cleaved caspase-3/GFAP (**A**) and TUNEL/GFAP (**B**) double positive cells in the excitotoxically damaged cortex at different survival times. Data are shown as the percentage of cleaved caspase-3 positive cells showing GFAP colocalization (A) ($n = 4$ for each survival time), and percentage of TUNEL positive cells showing GFAP colocalisation (B) ($n = 3$ for each survival time). GFAP-positive astrocytes are the majority of cleaved caspase-3 positive cells at 4 h and 3–7 days postlesion (A). Lower percentages are found at 10 h and 1 day postlesion. Decreased immunoreactivity for GFAP within the lesion core (see results) found at these times may account for the reduction in the amount of double positive cells. In contrast, GFAP/TUNEL-positive cells are a minority of TUNEL-positive cells at all survival times. However, percentage increases with time and in the cortical glial scar at 7 days postlesion, when the densitiy of TUNEL-positive cells at this timepoint is strongly diminished (see Fig. 2), GFAP/TUNEL-positive account for 26% of all TUNEL-positive nuclei. IR, immunoreactivity.

ally show TUNEL-positive nuclei, reactive astrocytes were frequently seen surrounding TUNEL-positive nuclei with their projections (Figs. 7G–I). At longer survival times, TUNEL staining was strongly reduced and was not found in the majority of scar forming GFAP-positive astrocytes (Fig. 7J). However, some reactive astrocytes located in the upper and more superficial glial scar and in the medial cortex showed TUNEL-positive nuclei (Figs. 7K–L), representing 26% of the total number of TUNEL-positive cells identified (Fig. 5). It should be noted that the number of TUNEL-positive cells at this time is strongly reduced (Fig. 2). Analysis of double-labeling for TUNEL and the oligodendroglial marker APC showed few if any TUNEL-positive oligodendrocytes throughout all survival times analyzed (Fig. 7M).

DISCUSSION

This study shows that following excitotoxic cortical damage to the postnatal rat brain, cleaved caspase-3 can be observed in some neuronal cells, the main cell type undergoing apoptotic cell death, as classicaly reported. However, the majority of cleaved caspase-3 was found in the nuclei of activated astrocytes within the lesioned hemisphere from early times and until glial scar formation. Neither microglial cells nor oligodendrocytes showed cleaved caspase-3 immunoreactivity. Interestingly, astroglial caspase-3 cleavage did not generally correlate with TUNEL labeling and apoptotic astrocytes were only seen at specific times and regions.

Astroglial cells can undergo cell death *in vitro* in a variety of situations such as calcium overload, oxidative stress, mitochondrial dysfunction, and treatment with apoptotic inducers like staurosporine and ceramide (Giffard and Swanson, 2005; Takuma et al., 2004), although it has been established that they are more resistant than neuronal cells (Xu et al., 2004). In this regard, studies of astroglial cell death after different types of acute injuries such as traumatic brain injury (Beer et al., 2000; Newcomb et al., 1999) and ischemia/excitotoxicity (Biran et al., 2006; Dihne et al., 2001), have shown that apoptosis occurs only in few astrocytes, in agreement with our findings in the injured neonatal brain. Alternatively, non-apoptotic roles of caspase-3 need to be considered.

Cleaved Caspase-3 is Found in the Astroglial Nuclei

The nuclear localization of cleaved caspase-3 in astrocytes has not only been previously demonstrated in injury paradigms (Acarin et al., 2005; Benjelloun et al., 2003; Johnson et al., 2005), but also in astrocytes of the normal adult CNS (Noyan-Ashraf et al., 2005) and in differentiating Bergmann glial cells (Oomman et al., 2005). It is largely established that during apoptosis, caspase-3 is the main protease responsible for the cleavage of nuclear proteins, like Poly(ADP-ribose) Polymerase-1 (PARP-1), acinus, and lamins (Eldadah and Faden, 2000), inducing DNA damage and chromatin condensation. To date, however, very little is known about the function of caspase-cleaved nuclear proteins that are independent of apoptosis, but recent reports have suggested several possible mechanisms. First, caspase-generated

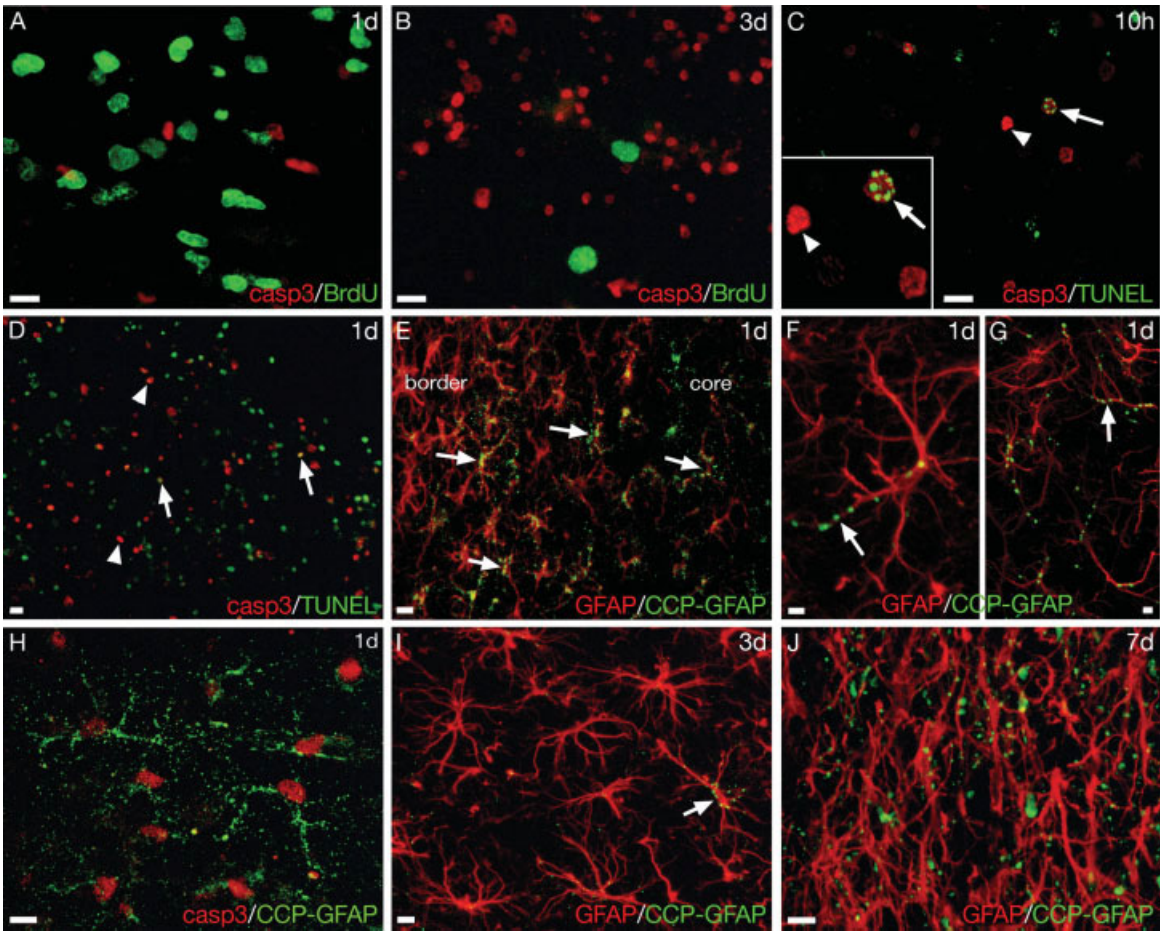


Fig. 6. Colocalization of cleaved caspase-3 with BrdU, TUNEL, and CCP-GFAP immunolabeling at several days (d) postlesion. Cleaved caspase-3 (casp3) (**A**-**D** and **H**) and GFAP (**E**-**G**, **I**, and **J**) are shown in red. BrdU (A, B), TUNEL (C, D), and CCP-GFAP (E-J) are shown in green. No colocalization is observed between cleaved caspase-3 and BrdU incorporation (A, B). Colocalization between cleaved caspase-3 and TUNEL labeling is seen in some cells (arrows in C and D), but TUNEL-negative and cleaved caspase-3-positive cells are more frequent (arrowheads in C and D). At 10 h and 1 day postlesion CCP-GFAP is seen in GFAP-positive astrocytes in the lesion core and adjacent border (arrows in E). CCP-GFAP shows a beaded immunoreactivity associated to astroglial projections which usually display low GFAP content (arrows in F and G). CCP-GFAP colocalizes with cleaved caspase-3 labeling within the lesion core (H). At 3 days, CCP-GFAP labeling is clearly diminished and only few immunoreactive astrocytes remain (arrow in I). No colocalization is seen at longer survival times (J). Scale bars = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

fragments of PARP-1 can interact with Nuclear Factor kappa B (NFkappaB) subunits and enhance NFkappaB transcriptional activity (Lamkanfi et al., 2006), a transcription factor present in the nucleus of postnatal reactive astrocytes (Acarin et al., 2000b). Second, cleavage of FoxO transcription factors, like FoxO3a, that contain a conserved caspase-3 cleavage site (Charvet et al., 2003), could also play a role in the modulation of gene expression during glial differentiation or astrogliosis. Third, it has been suggested that caspase-3 could serve as a mechanism of protein degradation required for the cellular response to changes in extrinsic signals (McLaughlin, 2004).

Presence of Caspase-Cleaved GFAP

One of the main findings of the present study was the detection of beaded caspase-cleaved GFAP immunoreactivity in astrocytes, colocalizing with cleaved caspase-3, within the lesion core at 10–24 h postlesion, when caspase-3 enzymatic activity was observed. Decreased GFAP immunoreactivity induced by excitotoxicity and ischemia within the lesion core during the first 24 h postlesion is a well characterized event, both in the immature (Acarin et al., 1999b), adult (Dihne et al., 2001), and aged brain (Castillo-Ruiz et al., 2007). As caspase-generated GFAP fragments are not recognized by commonly used GFAP antibodies directed to the full length molecule (Mouser et al., 2006), caspase-mediated cleavage of GFAP could, at least in part, explain the decrease in GFAP immunoreactivity observed in the lesion core at early times in several injury paradigms. This decrease in GFAP, together with the existence of astrocytes showing low GFAP content in gray matter (Walz, 2000), obviously leads an underestimation of the number of GFAP-positive astrocytes showing caspase-3 at early times (see Fig. 5).

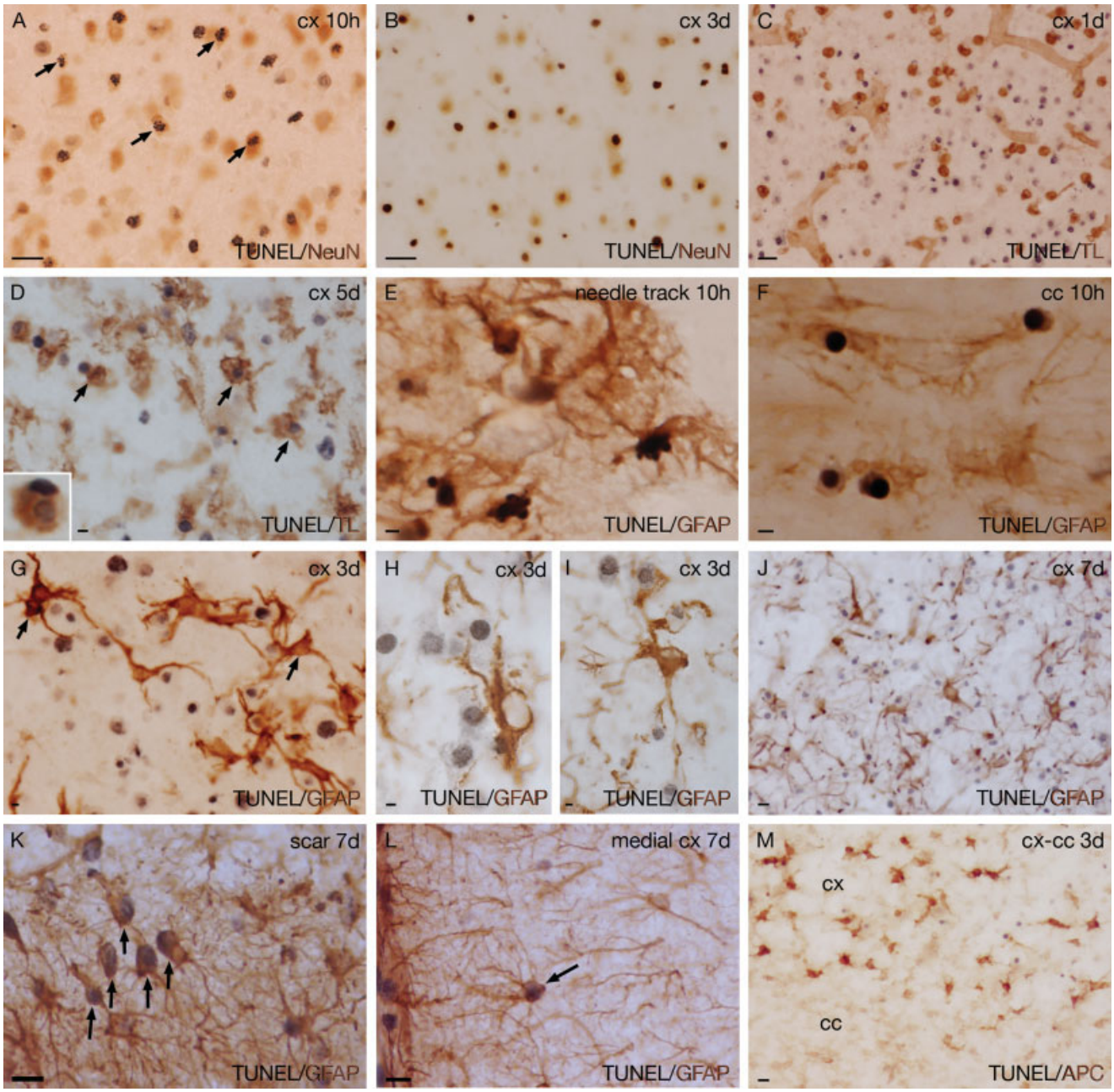


Fig. 7. Identification of TUNEL-positive cells at different survival times following a cortical excitotoxic lesion. TUNEL labeling is shown in gray-black (**A**-**M**) and NeuN (A, B), Tomato lectin (TL) (C, D), GFAP (E-L), or APC (M) are shown in brown. The majority of TUNEL-positive cells show NeuN labeling at different survival times (A, B). At 10 h and 1 day postlesion, TUNEL/NeuN double positive cells commonly display fragmented nuclei (arrows in A). Double staining for TUNEL and TL shows that whereas no colocalization is found at early times (C), some macrophages at 5–7 days postlesion display TUNEL-positive nuclei and/or TUNEL-positive material within the cytoplasm (inset in D). In TUNEL/GFAP double labeled sections, scattered double positive cells are found at 10 h in the needle track (E) and adjacent corpus callosum (F). At days 1–5 postlesion, no double positive cells are found (G-J), although close proximity between processes of GFAP-positive astrocytes and TUNEL-positive nuclei is often observed (arrows in G and H, I). At longer survival times, in the glial scar, scattered astrocytes located in the upper cortical layers (arrows in K) and the medial cortex (arrow in L) do show TUNEL-positive nuclei. No colocaization is seen between TUNEL and the oligodendroglial marker APC (M). Cc, corpus callosum; cx, cortex. Scale bars = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

In Alzheimer' disease brain (AD) and mouse models of AD, caspase-cleaved GFAP colocalizes with cleaved caspase-3 in beaded and/or fragmented processes of astrocytes located in plaque-rich regions and near blood vessels (Mouser et al., 2006), a process thought to be associated with cytoskeletal dismantling and cell death. However, in this model of acute postnatal injury, it is unclear if cleavage of GFAP is associated with cell death or contributes to cytoskeletal remodeling and associative morphological changes. In this regard, it should be noted that from 3 days postlesion, cleaved caspase-3 was found in hypertrophied reactive astrocytes that did not immu-

nolabelled for caspase-cleaved GFAP. Therefore, an interesting hypothesis would be that caspase-3 is participating in astrocyte cytoskeletal remodeling processes, a characteristic feature of astrogliosis, by cleaving intermediate filament proteins at specific sites. In support of this, we found that a majority of the reactive astrocytes expressing vimentin also were positive for cleaved caspase-3. Vimentin is specifically cleaved by caspase-3, irreversibly dismantling intermediate filaments (Byun et al., 2001), which may impact the integrity and dynamics of intracellular structures (Morishima, 1999). Similarly, the GFAP sequence contains a single caspase consensus sequence, $DLTD^{266}$, based on the general tetrapeptide motif DXXD recognized by caspases, and the antibody CCP-GFAP used in this study was synthesized to the downstream neoepitope that would be generated after caspase cleavage of full length GFAP (Mouser et al., 2006).

Other Non-Apoptotic Roles of Caspase-3

An important conclusion of this study is that cleaved caspase-3 is found in surviving reactive astrocytes, supporting the hypothesis of a non-apoptotic role of astroglial cleaved caspase-3, as has been postulated in other cell types, where non-apoptotic activities of caspases include the regulation of cell proliferation and differentiation (for review see McLaughlin, 2004; Schwerk and Schulze-Osthoff, 2003). One of the first described non-apoptotic roles of caspase-3 was the demonstration that caspase-3 cleavage is a physiological step during T lymphocyte activation and mitogenic induction (Miossec et al., 1997; Wilhelm et al., 1998). Recently, caspase-3 was characterized as a cell cycle-regulated protein considered a mitotic check point in HeLa cells (Hsu et al., 2006).

In the CNS, caspase activation may also play a role in neuronal cell dispersion and correct morphology (Rohn et al., 2004), and non-apoptotic neuronal caspase-3 cleavage occurs during normal hippocampal neuronal function such as in long-term potentiation processes (Dash et al., 2000), and in the soma and nerve endings of the adult brain, suggesting a contribution of this caspase to the regulation of synaptic plasticity (Shimohama et al., 2001). In addition, caspase-3 activation and substrate cleavage has been reported in the absence of cell death in gerbil hypoxia (Garnier et al., 2004) and in ischemic tolerance where neuronal caspase-3 is essential for the neuroprotective effect of preconditioning (Garnier et al. 2003; McLaughlin et al., 2003; Tanaka et al., 2004). Furthermore, other studies have also demonstrated a role of caspase-3 in the maturation of cerebellar granular cells during development (Oomman et al., 2004) and the differentiation of neural progenitor cells in the olfactory bulb (Fernando et al., 2005; Yan et al., 2001). Progenitor cells that divide and migrate through the rostral migratory stream en route to the olfactory bulb, show cleaved caspase-3 but no signs of cell death (Yan et al., 2001). In the present study, despite the fact that astroglial cells do undergo proliferation in this model of postnatal excitotoxicity (unpublished findings) no association was found between nuclear caspase-3 and BrdU incorporation.

With regards to glial cell differentiation, Oomann and coworkers, who have described cleaved caspase-3 in Bergmann glia during postnatal development in the absence of apoptotic or proliferation markers, have demonstrated a role of this protease in differentiation processes (Oomman et al., 2004, 2005, 2006). In addition, it was recently shown that constitutive non-apoptotic expression of the cleaved form of caspase-3 occurs in the nuclei of a subpopulation of astrocytes in the cerebellar cortex, hippocampus, and spinal cord of adult rats of different strains, which show expression of the sodium dependent glutamate transporter (EAAT1, GLAST) (Noyan-Ashraf et al., 2005). In relation to this, it was recently reported that the other glutamate transporter EAAT2, also expressed predominantly in astrocytes, is cleaved by caspase-3, leading to a drastic and selective inhibition of the transporter (Boston-Howes et al., 2006). In view of all these findings, it is becoming evident that caspase-3 may have unique, nontraditional roles in astrocytes that may be important to determine changes in cell phenotyping either during differentiation or after injury.

In conclusion, this study demonstrates for the first time that immature brain damage causes astroglial caspase-3 cleavage without associated apoptotic death. Alternative roles for capase 3 were examined, but no correlation was seen between caspase-3 and cell proliferation. Instead, this work suggests a novel role for cleaved caspase-3 in the cytoskeletal remodeling associated with astrogliosis.

ACKNOWLEDGMENTS

We would like to thank M.A. Martil for his excellent technical help, E. Sanz for helping in the activity assay, and M. Roldan from the Servei Microscopia for confocal microscopy assistance. SV and MF hold a FI fellowship from the Autonomous University of Barcelona, Spain.

REFERENCES

Acarin L, Gonzalez B, Castellano B. 2000a. Neuronal, astroglial and microglial cytokine expression after an excitotoxic lesion in the immature rat brain. Eur J Neurosci 12:3505–3520.

Acarin L, Gonzalez B, Castellano B. 2000b. STAT3 and NFkappaB activation precedes glial reactivity in the excitotoxically injured young cortex but not in the corresponding distal thalamic nuclei. J Neuropathol Exp Neurol 59:151–163.

Acarin L, Gonzalez B, Castellano B, Castro AJ. 1996. Microglial response to N-methyl-D-aspartate-mediated excitotoxicity in the immature rat brain. J Comp Neurol 367:361–374.

Acarin L, González B, Castro AJ, Castellano B. 1999a. Primary cortical glial reaction versus secondary thalamic glial response in the excitotoxically injured young brain. Microglial/macrophage response and MHC class I, II expression. Neuroscience 89:549–565.

Acarin L, González B, Hidalgo J, Castro AJ, Castellano B. 1999b. Primary cortical glial reaction versus secondary thalamic glial response in the excitotoxically injured young brain. Astroglial response and metallothionein expression. Neuroscience 92:827–839.

Acarin L, Peluffo H, Barbeito L, Castellano B, Gonzalez B. 2005. Astroglial nitration after postnatal excitotoxic damage: Correlation with nitric oxide sources, cytoskeletal, apoptotic and antioxidant proteins. J Neurotrauma 22:189–200.

Acarin L, Peluffo H, Gonzalez B, Castellano B. 2002. Expression of inducible nitric oxide synthase and cyclooxygenase-2 after excitotoxic damage to the immature rat brain. J Neurosci Res 68:745–754.

Allen JW, Knoblach SM, Faden AI. 1999. Combined mechanical trauma and metabolic impairment in vitro induces NMDA receptor-dependent neuronal cell death and caspase-3-dependent apoptosis. Faseb J 13:1875–1882.

Beer R, Franz G, Srinivasan A, Hayes RL, Pike BR, Newcomb JK, Zhao X, Schmutzhard E, Poewe W, Kampfl A. 2000. Temporal profile and cell subtype distribution of activated caspase-3 following experimental traumatic brain injury. J Neurochem 75:1264–1273.

Benjelloun N, Joly LM, Palmier B, Plotkine M, Charriaut-Marlangue C. 2003. Apoptotic mitochondrial pathway in neurones and astrocytes after neonatal hypoxia-ischaemia in the rat brain. Neuropathol Appl Neurobiol 29:350–360.

Biran V, Joly LM, Heron A, Vernet A, Vega C, Mariani J, Renolleau S, Charriaut-Marlangue C. 2006. Glial activation in white matter following ischemia in the neonatal P7 rat brain. Exp Neurol 199:103–112.

Boston-Howes W, Gibb SL, Williams EO, Pasinelli P, Brown RH Jr, Trotti D. 2006. Caspase-3 cleaves and inactivates the glutamate transporter EAAT2. J Biol Chem 281:14076–14084.

Brecht S, Gelderblom M, Srinivasan A, Mielke K, Dityateva G, Herdegen T. 2001. Caspase-3 activation and DNA fragmentation in primary hippocampal neurons following glutamate excitotoxicity. Brain Res Mol Brain Res 94:25–34.

Byun Y, Chen F, Chang R, Trivedi M, Green KJ, Cryns VL. 2001. Caspase cleavage of vimentin disrupts intermediate filaments and promotes apoptosis. Cell Death Differ 8:443–450.

Castillo-Ruiz MM, Campuzano O, Acarin L, Castellano B, Gonzalez B. 2007. Delayed neurodegeneration and early astrogliosis after excitotoxicity to the aged brain. Exp Gerontol 42:343–354.

Charvet C, Alberti I, Luciano F, Jacquel A, Bernard A, Auberger P, Deckert M. 2003. Proteolytic regulation of Forkhead transcription factor FOXO3a by caspase-3-like proteases. Oncogene 22:4557–4568.

Chen J, Nagayama T, Jin K, Stetler RA, Zhu RL, Graham SH, Simon RP. 1998. Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. J Neurosci 18:4914–4928.

Dash PK, Blum S, Moore AN. 2000. Caspase activity plays an essential role in long-term memory. Neuroreport 11:2811–2816.

Dihne M, Block F, Korr H, Topper R. 2001. Time course of glial proliferation and glial apoptosis following excitotoxic CNS injury. Brain Res 902:178–189.

Du Y, Bales KR, Dodel RC, Hamilton-Byrd E, Horn JW, Czilli DL, Simmons LK, Ni B, Paul SM. 1997. Activation of a caspase 3-related cysteine protease is required for glutamate-mediated apoptosis of cultured cerebellar granule neurons. Proc Natl Acad Sci USA 94: 11657–11662.

Eldadah BA, Faden AI. 2000. Caspase pathways, neuronal apoptosis, and CNS injury. J Neurotrauma 17:811–29.

Fernando P, Brunette S, Megeney LA. 2005. Neural stem cell differentiation is dependent upon endogenous caspase 3 activity. Faseb J 19:1671–1673.

Ferriero DM. 2004. Neonatal brain injury. N Engl J Med 351:1985–1995.

Garnier P, Prigent-Tessier A, Van Hoecke M, Bertrand N, Demougeot C, Sordet O, Swanson RA, Marie C, Beley A. 2004. Hypoxia induces caspase-9 and caspase-3 activation without neuronal death in gerbil brains. Eur J Neurosci 20:937–946.

Garnier P, Ying W, Swanson RA. 2003. Ischemic preconditioning by caspase cleavage of poly (ADP-ribose) polymerase-1. J Neurosci 23: 7967–73.

Giffard RG, Swanson RA. 2005. Ischemia-induced programmed cell death in astrocytes. Glia 50:299–306.

Hsu SL, Yu CT, Yin SC, Tang MJ, Tien AC, Wu YM, Huang CY. 2006. Caspase 3, periodically expressed and activated at G2/M transition, is required for nocodazole-induced mitotic checkpoint. Apoptosis 11:765–771.

Johnson EA, Svetlov SI, Wang KK, Hayes RL, Pineda JA. 2005. Cell-specific DNA fragmentation may be attenuated by a survivin-dependent mechanism after traumatic brain injury in rats. Exp Brain Res 167:17–26.

Lamkanfi M, Declercq W, Vanden Berghe T, Vandenabeele P. 2006. Caspases leave the beaten track: Caspase-mediated activation of NF-kappaB. J Cell Biol 173:165–171.

Manabat C, Han BH, Wendland M, Derugin N, Fox CK, Choi J, Holtzman DM, Ferriero DM, Vexler ZS. 2003. Reperfusion differentially induces caspase-3 activation in ischemic core and penumbra after stroke in immature brain. Stroke 34:207–213.

Marks N, Berg MJ. 1999. Recent advances on neuronal caspases in development and neurodegeneration. Neurochem Int 35:195–220.

McLaughlin B. 2004. The kinder side of killer proteases: Caspase activation contributes to neuroprotection and CNS remodeling. Apoptosis 9:111–121.

McLaughlin B, Hartnett KA, Erhardt JA, Legos JJ, White RF, Barone FC, Aizenman E. 2003. Caspase 3 activation is essential for neuroprotection in preconditioning. Proc Natl Acad Sci USA 100:715–720.

Miossec C, Dutilleul V, Fassy F, Diu-Hercend A. 1997. Evidence for CPP32 activation in the absence of apoptosis during T lymphocyte stimulation. J Biol Chem 272:13459–13462.

Morishima N. 1999. Changes in nuclear morphology during apoptosis correlate with vimentin cleavage by different caspases located either upstream or downstream of Bcl-2 action. Genes Cells 4:401–414.

Mouser PE, Head E, Ha KH, Rohn TT. 2006. Caspase-mediated cleavage of glial fibrillary acidic protein within degenerating astrocytes of the Alzheimer's disease brain. Am J Pathol 168:936–946.

Namura S, Zhu J, Fink K, Endres M, Srinivasan A, Tomaselli KJ, Yuan J, Moskowitz MA. 1998. Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. J Neurosci 18: 3659–3668.

Narkilahti S, Pirttila TJ, Lukasiuk K, Tuunanen J, Pitkanen A. 2003. Expression and activation of caspase 3 following status epilepticus in the rat. Eur J Neurosci 18:1486–1496.

Nath R, Scott M, Nadimpalli R, Gupta R, Wang KK. 2000. Activation of apoptosis-linked caspase(s) in NMDA-injured brains in neonatal rats. Neurochem Int 36:119–126.

Newcomb JK, Zhao X, Pike BR, Hayes RL. 1999. Temporal profile of apoptotic-like changes in neurons and astrocytes following controlled cortical impact injury in the rat. Exp Neurol 158:76–88.

Nottingham SA, Springer JE. 2003. Temporal and spatial distribution of activated caspase-3 after subdural kainic acid infusions in rat spinal cord. J Comp Neurol 464:463–471.

Noyan-Ashraf MH, Brandizzi F, Juurlink BH. 2005. Constitutive nuclear localization of activated caspase 3 in subpopulations of the astroglial family of cells. Glia 49:588–593.

Oomman S, Finckbone V, Dertien J, Attridge J, Henne W, Medina M, Mansouri B, Singh H, Strahlendorf H, Strahlendorf J. 2004. Active caspase-3 expression during postnatal development of rat cerebellum is not systematically or consistently associated with apoptosis. J Comp Neurol 476:154–173.

Oomman S, Strahlendorf H, Dertien J, Strahlendorf J. 2006. Bergmann glia utilize active caspase-3 for differentiation. Brain Res 1078:19–34.

Oomman S, Strahlendorf H, Finckbone V, Strahlendorf J. 2005. Non-lethal active caspase-3 expression in Bergmann glia of postnatal rat cerebellum. Brain Res Dev Brain Res 160:130–45.

Pulera MR, Adams LM, Liu H, Santos DG, Nishimura RN, Yang F, Cole GM, Wasterlain CG. 1998. Apoptosis in a neonatal rat model of cerebral hypoxia-ischemia. Stroke 29:2622–2630.

Rohn TT, Cusack SM, Kessinger SR, Oxford JT. 2004. Caspase activation independent of cell death is required for proper cell dispersal and correct morphology in PC12 cells. Exp Cell Res 295:215–225.

Schwerk C, Schulze-Osthoff K. 2003. Non-apoptotic functions of caspases in cellular proliferation and differentiation. Biochem Pharmacol 66:1453–1458.

Shimohama S, Tanino H, Fujimoto S. 2001. Differential subcellular localization of caspase family proteins in the adult rat brain. Neurosci Lett 315:125–128.

Springer JE, Nottingham SA, McEwen ML, Azbill RD, Jin Y. 2001. Caspase-3 apoptotic signaling following injury to the central nervous system. Clin Chem Lab Med 39:299–307.

Su JH, Nichol KE, Sitch T, Sheu P, Chubb C, Miller BL, Tomaselli KJ, Kim RC, Cotman CW. 2000. DNA damage and activated caspase-3 expression in neurons and astrocytes: Evidence for apoptosis in frontotemporal dementia. Exp Neurol 163:9–19.

Takuma K, Baba A, Matsuda T. 2004. Astrocyte apoptosis: implications for neuroprotection. Prog Neurobiol 72:111–127.

Tanaka H, Yokota H, Jover T, Cappuccio I, Calderone A, Simionescu M, Bennett MV, Zukin RS. 2004. Ischemic preconditioning: neuronal survival in the face of caspase-3 activation. J Neurosci 24:2750–2759.

Tenneti L, Lipton SA. 2000. Involvement of activated caspase-3-like proteases in N-methyl-D-aspartate-induced apoptosis in cerebrocortical neurons. J Neurochem 74:134–142.

Vannucci SJ, Hagberg H. 2004. Hypoxia-ischemia in the immature brain. J Exp Biol 207(Part 18):3149–3154.

Walz W. 2000. Controversy surrounding the existence of discrete functional classes of astrocytes in adult gray matter. Glia 31:95–103.

Wilhelm S, Wagner H, Hacker G. 1998. Activation of caspase-3-like enzymes in non-apoptotic T cells. Eur J Immunol 28:891–900.

Xu L, Chock VY, Yang EY, Giffard RG. 2004. Susceptibility to apoptosis varies with time in culture for murine neurons and astrocytes: Changes in gene expression and activity. Neurol Res 26:632–643.

Yan XX, Najbauer J, Woo CC, Dashtipour K, Ribak CE, Leon M. 2001. Expression of active caspase-3 in mitotic and postmitotic cells of the rat forebrain. J Comp Neurol 433:4–22.

ARTICLE II

"*Distinct spatial and temporal activation profile of apoptotic pathways in neurons and glial cells after excitotoxic damage in the neonatal rat brain*"

J. Neuroscience Research. *In press* (2007).

Distinct Spatial and Temporal Activation of Caspase Pathways in Neurons and Glial Cells After Excitotoxic Damage to the Immature Rat Brain

ABSTRACT

Although cleaved caspase-3 is known to be involved in apoptotic cell death mechanisms in neurons, it can also be involved in a nonapoptotic role in astrocytes after postnatal excitotoxic injury. Here we evaluate participation of upstream pathways activating caspase-3 in neurons and glial cells, by studying the intrinsic pathway via caspase-9, the extrinsic pathway via caspase-8, and activation of the p53-dependent pathway. Nmethyl-D-aspartate (NMDA) was injected intracortically in 9-day-old postnatal rats, which were sacrificed at several survival times between 4 hr postlesion (pl) and 7 days pl. We analyzed temporal and spatial expression of caspase-8, caspase-9, and p53 and correlation with neuronal and glial markers and caspase-3 activation. Caspase-9 was significantly activated at 10 hpl, strongly correlating with caspase-3. It was present mainly in damaged cortical and hippocampal neurons but was also seen in astrocytes and oligodendrocytes in layer VI and corpus callosum (cc). Caspase-8 showed a diminished correlation with caspase-3. It was present in cortical neurons at 10 - 72 hpl, showing layer specificity, and also in astroglial and microglial nuclei, mainly in layer VI and cc. p53 Expression increased at 10 - 72 hpl but did not correlate with caspase-3. p53 was seen in neurons of the degenerating cortex and in some astrocytes and microglial cells of layer VI and cc. In conclusion, after neonatal excitotoxicity, mainly the mitochondrial intrinsic pathway mediates neuronal caspase-3 and cell death. In astrocytes, caspase-3 is not widely correlated with caspase-8, caspase-9, or p53, except in layer VI-cc astrocytes, where activation of upstream cascades occurs.

Journal of Neuroscience Research

Copy of e-mail Notification

Your article (21450) from "Journal of Neuroscience Research" is available for download
=====
Journal of Neuroscience Research Published by John Wiley & Sons, Inc.

Dear Author,

Your article page proofs for Journal of Neuroscience Research are ready for review. John Wiley & Sons has made this article available to you online for faster, more efficient editing. Please follow the instructions below and you will be able to access a PDF version of your article as well as relevant accompanying paperwork.

First, make sure you have a copy of Adobe Acrobat Reader software to read these files. This is free software and is available for user downloading at http://www.adobe.com/products/acrobat/readstep.html.

Open your web browser, and enter the following web address:
http://kwglobal.co.in/jw/retrieval.aspx

You will be prompted to log in, and asked for a password. Your login name will be your email address, and your password will be ----

Example:

Login: your e-mail address
Password: ----

The site contains one file, containing:

- Author Instructions Checklist
- Adobe Acrobat Users - NOTES tool sheet
- Reprint Order form
- A copy of your page proofs for your article

Print out this file, and fill out the forms by hand. (If you do not wish to order reprints, please mark a "0" on the reprint order form.) Read your page proofs carefully and:

- indicate changes or corrections in the margin of the page proofs
- answer all queries (footnotes A,B,C, etc.) on the last page of the PDF proof
- proofread any tables and equations carefully
- check your figure legends for accuracy

Within 48 hours, please return via fax or express mail all materials to the address given below. This will include:

1) Page proofs with corrections
2) Reprint Order form

Return to:

Journal of Neuroscience Research

Copy of e-mail Notification

Jeremy Hargrove
Production Editor
John Wiley & Sons, Inc.
111 River Street, Hoboken, NJ 07030

Phone: 201-748-5966
Fax: 201-748-6052
Email: JHargrov@wiley.com

Technical problems? If you experience technical problems downloading your file or any other problem with the website listed above, please contact Birender/Sundeep (e-mail: wileysupport@kwglobal.com, phone: +91 (44) 42058888 ext. 310).

Questions regarding your article? Please don't hesitate to contact me with any questions about the article itself, or if you have trouble interpreting any of the questions listed at the end of your file. REMEMBER TO INCLUDE YOUR ARTICLE NO. (21450) WITH ALL CORRESPONDENCE. This will help both of us address your query most efficiently.

As this e-proofing system was designed to make the publishing process easier for everyone, we welcome any and all feedback. Thanks for participating in our e-proofing system!

This e-proof is to be used only for the purpose of returning corrections to the publisher.

Sincerely,

Jeremy Hargrove
Production Editor
John Wiley & Sons, Inc.
111 River Street, Hoboken, NJ 07030

Phone: 201-748-5966
Fax: 201-748-6052
Email: JHargrov@wiley.com

WILEY
Publishers Since 1807

111 River Street, Hoboken, NJ 07030

Journal of Neuroscience Research Production

*****IMMEDIATE RESPONSE REQUIRED*****
Please follow these instructions to avoid delay of publication.

☐ **READ PROOFS CAREFULLY**

- This will be your only chance to review these proofs.
- Please note that the volume and page numbers shown on the proofs are for position only.

☐ **ANSWER ALL QUERIES ON PROOFS** (Queries for you to answer are attached as the last page of your proof.)

- Mark all corrections directly on the proofs. Note that excessive author alterations may ultimately result in delay of publication and extra costs may be charged to you.

☐ **CHECK FIGURES AND TABLES CAREFULLY**

- Check size, numbering, and orientation of figures.
- All images in the PDF are downsampled (reduced to lower resolution and file size) to facilitate Internet delivery. These images will appear at higher resolution and sharpness in the printed article.

☐ **COMPLETE REPRINT ORDER FORM**

- Fill out the attached reprint order form. It is important to return the form even if you are not ordering reprints. Reprints will be shipped 4-6 weeks after your article appears in print.

☐ **ADDITIONAL COPIES**

- If you wish to purchase additional copies of the journal in which your article appears, please contact Kim Lloyd at (201) 748-8839, fax (201) 748-6021, or E-mail at klloyd@wiley.com

RETURN WITHIN 48 HOURS OF RECEIPT VIA FAX TO
201-748-6052
or direct questions to:

Jeremy Hargrove, Production Editor
Journal of Neuroscience Research
Phone: 201-748-5966
Refer to journal acronym (JNR) and article production number

Softproofing for advanced Adobe Acrobat Users - NOTES tool

NOTE: ACROBAT READER FROM THE INTERNET DOES NOT CONTAIN THE NOTES TOOL USED IN THIS PROCEDURE.

Acrobat annotation tools can be very useful for indicating changes to the PDF proof of your article. By using Acrobat annotation tools, a full digital pathway can be maintained for your page proofs.

The NOTES annotation tool can be used with either Adobe Acrobat 6.0 or Adobe Acrobat 7.0. Other annotation tools are also available in Acrobat 6.0, but this instruction sheet will concentrate on how to use the NOTES tool. Acrobat Reader, the free Internet download software from Adobe, DOES NOT contain the NOTES tool. In order to softproof using the NOTES tool you must have the full software suite Adobe Acrobat Exchange 6.0 or Adobe Acrobat 7.0 installed on your computer.

Steps for Softproofing using Adobe Acrobat NOTES tool:

1. Open the PDF page proof of your article using either Adobe Acrobat Exchange 6.0 or Adobe Acrobat 7.0. Proof your article on-screen or print a copy for markup of changes.

2. Go to Edit/Preferences/Commenting (in Acrobat 6.0) or Edit/Preferences/Commenting (in Acrobat 7.0) check “Always use login name for author name” option. Also, set the font size at 9 or 10 point.

3. When you have decided on the corrections to your article, select the NOTES tool from the Acrobat toolbox (Acrobat 6.0) and click to display note text to be changed, or Comments/Add Note (in Acrobat 7.0).

4. Enter your corrections into the NOTES text box window. Be sure to clearly indicate where the correction is to be placed and what text it will effect. If necessary to avoid confusion, you can use your TEXT SELECTION tool to copy the text to be corrected and paste it into the NOTES text box window. At this point, you can type the corrections directly into the NOTES text box window. **DO NOT correct the text by typing directly on the PDF page.**

5. Go through your entire article using the NOTES tool as described in Step 4.

6. When you have completed the corrections to your article, go to Document/Export Comments (in Acrobat 6.0) or Comments/Export Comments (in Acrobat 7.0). Save your NOTES file to a place on your harddrive where you can easily locate it. **Name your NOTES file with the article number assigned to your article in the original softproofing e-mail message.**

7. **When closing your article PDF be sure NOT to save changes to original file.**

8. To make changes to a NOTES file you have exported, simply re-open the original PDF proof file, go to Document/Import Comments and import the NOTES file you saved. Make changes and reexport NOTES file keeping the same file name.

9. When complete, attach your NOTES file to a reply e-mail message. Be sure to include your name, the date, and the title of the journal your article will be printed in.

C1

REPRINT BILLING DEPARTMENT ∉ 111 RIVER STREET ∉ HOBOKEN, NJ 07030
PHONE: (201) 748-8789; FAX: (201) 748-6326
E-MAIL: reprints@wiley.com

PREPUBLICATION REPRINT ORDER FORM

Please complete this form even if you are not ordering reprints. This form **MUST** be returned with your corrected proofs and original manuscript. Your reprints will be shipped approximately 4 weeks after publication. Reprints ordered after printing will be substantially more expensive.

JOURNAL **Journal of Neuroscience Research** VOLUME _____ ISSUE _____

TITLE OF MANUSCRIPT _____

MS. NO. JNR- NO. OF PAGES AUTHOR(S)

No. of Pages	100 Reprints	200 Reprints	300 Reprints	400 Reprints	500 Reprints
	$	$	$	$	$
1-4	**336**	**501**	**694**	**890**	**1052**
5-8	**469**	**703**	**987**	**1251**	**1477**
9-12	**594**	**923**	**1234**	**1565**	**1850**
13-16	**714**	**1156**	**1527**	**1901**	**2273**
17-20	**794**	**1340**	**1775**	**2212**	**2648**
21-24	**911**	**1529**	**2031**	**2536**	**3037**
25-28	**1004**	**1707**	**2267**	**2828**	**3388**
29-32	**1108**	**1894**	**2515**	**3135**	**3755**
33-36	**1219**	**2092**	**2773**	**3456**	**4143**
37-40	**1329**	**2290**	**3033**	**3776**	**4528**

**REPRINTS ARE ONLY AVAILABLE IN LOTS OF 100. IF YOU WISH TO ORDER MORE THAN 500 REPRINTS, PLEASE CONTACT OUR REPRINTS DEPARTMENT AT (201) 748-8789 FOR A PRICE QUOTE.

☐ Please send me _____ reprints of the above article at $ _____

Please add appropriate State and Local Tax for United States orders only. (Tax Exempt No._____) $ _____

Please add 5% Postage and Handling $ _____

TOTAL AMOUNT OF ORDER** $ _____

***International orders must be paid in currency and drawn on a U.S. bank*

Please check one: ☐ Check enclosed ☐ Bill me ☐ Credit Card

If credit card order, charge to: ☐ American Express ☐ Visa ☐ MasterCard

Credit Card No _____ Signature _____ Exp. Date _____

BILL TO:
Name _____
Institution _____
Address _____
_____

SHIP TO: (Please, no P.O. Box numbers)
Name _____
Institution _____
Address _____
_____

Purchase Order No. _____ Phone _____ Fax _____

E-mail

Journal of Neuroscience Research 00:000–000 (2007)

Distinct Spatial and Temporal Activation of Caspase Pathways in Neurons and Glial Cells After Excitotoxic Damage to the Immature Rat Brain

Sonia Villapol,* Laia Acarin, Maryam Faiz, Bernardo Castellano, and Berta Gonzalez

Medical Histology, Faculty of Medicine, Department of Cell Biology, Physiology and Immunology, Institute of Neurosciences, Autonomous University of Barcelona, Bellaterra, Spain

Although cleaved caspase-3 is known to be involved in apoptotic cell death mechanisms in neurons, it can also be involved in a nonapoptotic role in astrocytes after postnatal excitotoxic injury. Here we evaluate participation of upstream pathways activating caspase-3 in neurons and glial cells, by studying the intrinsic pathway via caspase-9, the extrinsic pathway via caspase-8, and activation of the p53-dependent pathway. N-methyl-D-aspartate (NMDA) was injected intracortically in 9-day-old postnatal rats, which were sacrificed at several survival times between 4 hr postlesion (pl) and 7 days pl. We analyzed temporal and spatial expression of caspase-8, caspase-9, and p53 and correlation with neuronal and glial markers and caspase-3 activation. Caspase-9 was significantly activated at 10 hpl, strongly correlating with caspase-3. It was present mainly in damaged cortical and hippocampal neurons but was also seen in astrocytes and oligodendrocytes in layer VI and corpus callosum (cc). Caspase-8 showed a diminished correlation with caspase-3. It was present in cortical neurons at 10–72 hpl, showing layer specificity, and also in astroglial and microglial nuclei, mainly in layer VI and cc. p53 Expression increased at 10–72 hpl but did not correlate with caspase-3. p53 Was seen in neurons of the degenerating cortex and in some astrocytes and microglial cells of layer VI and cc. In conclusion, after neonatal excitotoxicity, mainly the mitochondrial intrinsic pathway mediates neuronal caspase-3 and cell death. In astrocytes, caspase-3 is not widely correlated with caspase-8, caspase-9, or p53, except in layer VI-cc astrocytes, where activation of upstream cascades occurs.

Key words: neonatal; microglia; astrocyte; p53; oligodendrocyte

Caspases are cystein proteases expressed as inactive precursors that undergo proteolytic maturation into a large subunit (p20) and a small subunit (p10), which combine to form an active tetramer. Upon activation, initiator caspases such as caspase-8, -9, -10, and -2 cleave executioner caspases-3, -6, and -7, which are effector proteases degrading structural proteins, signaling molecules, and DNA repair enzymes (Marks and Berg, 1999; Salvesen and Dixit, 1999; Hengartner, 2000; Fischer et al., 2003; Kumar, 2007). Accordingly, activation of executioner caspases, mainly caspase-3, has been viewed as the terminal event in apoptosis. However, emerging evidence suggests a broader role for caspases in cell proliferation, cell cycle regulation, differentiation, and gene expression (Schwerk and Schulze-Osthoff, 2003; McLaughlin, 2004) and implies that the cell type and environment play an important role in defining the consequences of caspase activation.

The most common events ensuing from caspase-3 activation are the intrinsic and extrinsic pathways of apoptotic cell death through caspase-9 and caspase-8 activation, respectively. The mitochondrial intrinsic pathway is triggered by cellular stress signals such as DNA damage, ionic dysfunction, and subcellular relocalization of proapoptotic proteins. The key event in this pathway is mitochondrial cytochrome c release and its binding with APAF, which facilitates apoptosome formation that activates caspase-9 by proteolysis and dimerization, activating downstream effector caspase-3 (Costantini et al., 2002; Kumar, 2007). The extrinsic pathway can be induced by several stimuli that activate death receptors belonging to the tumor necrosis factor (TNF) family, including FAS receptor (CD95), TNF receptor-1

Contract grant number: BFU2005-02783; Contract grant number: Marato TV3-061710; Contract grant number: 2005SGR-00956. AQ1

*Correspondence to: Sonia Villapol, Medical Histology, Torre M5, Department Cell Biology, Physiology and Immunology and Institute of Neurosciences, Autonomous University of Barcelona, Bellaterra 08193, Barcelona, Spain. E-mail: sonia.villapol@uab.es

Received 30 March 2007; Revised 29 May 2007; Accepted 30 May 2007

Published online 00 Month 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.21450

(TNFR1), DR4, and DR5. Ligand binding to these receptors induces formation of a death-inducing signaling complex (DISC) that recruits procaspase-8 via binding to the adaptor protein Fas-associated death domain-containing protein (FADD). This recruitment results in caspase-8 activation by self-cleavage and oligomerization and subsequent activation of caspase-3 (Strasser et al., 2000; Thorburn, 2004; Kumar, 2007). Additionally, in certain cell types, the extrinsic pathway has the ability to cross-talk with the intrinsic pathway by caspase-8 cleavage of the Bcl-2 family member Bid. Truncated Bid (tBid) translocates to the mitochondria, where it induces cytochrome c release and caspase-9 activation, providing an amplification mechanism (Velier et al., 1999; Fulda and Debatin, 2004; Stefanis, 2005). Another molecule involved in the apoptotic cascade and caspase-3 activation is the tumor suppressor and transcription factor p53 (Lane, 1993). In addition to its role in cell cycle control, p53-dependent transcriptional activation of proapoptotic genes has been hypothesized to regulate cell death in response to DNA damage resulting from oxidative stress, a common pathological mechanism in excitotoxicity and ischemia (Sakhi et al., 1994, 1996; Didier et al., 1996; Renolleau et al., 1997; Mattson, 2003). p53 Induces nucleosomal fragmentation and activates transcription of Bax and other proapoptotic proteins involved in cytochrome c release from the mitochondria and caspase activation (Eizenberg et al., 1996).

We have previously described (Acarin et al., 2007) how, after excitotoxicity to the postnatal day 9 rat brain, active caspase-3 is found in some neuronal cells undergoing apoptotic cell death, as expected. However, widespread caspase-3 was observed mainly in reactive astrocytes, mainly in the absence of apoptotic signs. Caspase-3 activation was not seen, at any time, in microglia or oligodendrocytes. Accordingly, the aim of the present study is to investigate whether caspase-9, caspase-8, and p53 pathways are activated in caspase-3-negative cells (oligodendrocytes and microglia), caspase-3-positive cells undergoing apoptosis (neurons), and caspase-3-positive cells in the absence of cell death (astrocytes). For this purpose, we have analyzed the distribution, time course, and cell type showing expression of these molecules and their correlation with caspase-3 activation at several survival times postlesion.

MATERIALS AND METHODS

Excitotoxic Lesions and Tissue Processing

Nine-day-old Long Evans black-hooded rats pups of both sexes weighing 12–20 g (Janvier, France) were anesthetized with isoflurane (Baxter International Inc.) before receiving an intracerebral injection of 20 nmol N-methyl-D-aspartate (NMDA; Sigma-Aldrich, St. Louis, MO) diluted in 0.15 μl saline solution (0.9% NaCl) at a rate of 0.05 μl/min using an automatic injector. All intracerebral injections were made into the right sensorimotor cortex at the level of the coronal suture (2 mm lateral to bregma and at 0.5 mm depth) using a stereotaxic frame (Kopf Instruments), as described previously (Acarin et al., 1996). Control animals followed the same procedure, but received an injection of 0.15 μl saline solution. After 4, 10, 24, and 72 hr and 7 days postinjection, rats were anesthetized and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were postfixed for 4 hr and cryoprotected in 30% sucrose solution before being frozen with dry CO_2. Six NMDA- and two saline-injected animals were used for each of the five survival times. Controls aged 9, 10, 12, and 16 days were also used. Our experimental animal work was conducted according to Spanish regulations in agreement with European Union directives and was approved by the ethical commission of the Autonomous University of Barcelona.

Immunohistochemistry for Caspase-8, Caspase-9, and p53

Coronal sections (30-μm-thick) were obtained with a Leitz cryostat. Free-floating parallel sections were processed for endogenous peroxidase inactivation and blocked in 0.05 M Tris-buffered saline (TBS), pH 7.4, containing 10% fetal calf serum and 1% Triton X-100 for 1 hr. Afterward, sections were incubated overnight at 4°C with either one of the following primary antibodies: 1) mouse monoclonal antibody recognizing the proform and active form of caspase-8 (p20 subunit; 1:200, sc-5263; Santa Cruz Biotechnology, Santa Cruz, CA); 2) rabbit polyclonal antibody anti-active caspase-9 (p18 subunit; 1:200, catalog No. 3151-100; Biovision); 3) goat polyclonal antibody anti-p53 (1:200, sc-5263; Santa Cruz Biotechnology). As negative controls, sections were incubated in media lacking the primary antibody. After several washes, sections were incubated for 1 hr with either biotinylated anti-mouse secondary antibody (1:400, RPN1001V1; Amersham, Arlington Heights, IL; for caspase-8), biotinylated anti-rabbit secondary antibody (1:400, RPN1004V1; Amersham; for caspase-9), or biotinylated anti-goat secondary antibody (1:400, RPN1025V; Amersham; for p53). Specific labeling was visualized by incubation with avidin-peroxidase (1:400, P0364; Dakopatts) for 1 hr with a subsequent 3,3′-diaminobenzidine (DAB)-hydrogen peroxide developing procedure.

Double Immunofluorescence Labeling for Cleaved Caspase-3 and Cell-Specific Markers

For double labeling, immunohistochemistry for active caspase-8, active caspase-9, and p53 was carried out as described above but using cy3-conjugated anti-mouse (1:1,000, PA43002V; Amersham), cy3-conjugated anti-rabbit (1:1,000, PA430024V; Amersham), and cy3-conjugated anti-goat (1:1,000, 705-165-003; Jackson Immunoresearch, West Grove, PA) as secondary antibodies. After rinsing, sections were incubated with one of the following primary antibodies: 1) rabbit anticleaved caspase-3 (p17 or p19 subunit; 1:700, No. 9661; Cell Signaling, Beverly, MA); 2) rabbit antiglial fibrillary acidic protein (GFAP; 1:1,000, Z-0224; Dakopatts) for astroglial labeling; 3) monoclonal mouse antineuronal nuclear antigen (NeuN; 1:500, MAB377; Chemicon, Temecula, CA), for neuronal labeling; and 4) rabbit antiadenomatous polypopsis coli (APC; 1:500, OP80; Calbiochem, La Jolla, CA) as an oligodendroglial marker. Immunostaining was

J_ID: Z3P Customer A_ID: 21450 Cadmus Art: JNR21450 Date: 13-JULY-07 Stage: I Page: 3

visualized with either cy2-conjugated anti-rabbit secondary antibody (1:1,000, PA-42004; Amersham) or cy2-conjugated anti-mouse secondary antibody (1:1,000, PA-42004; Amersham). For microglial labeling, sections were processed for tomato lectin histochemistry by incubating with the biotinylated lectin from *Lycopersicon esculentum* (tomato lectin; 1:150, L-9389; Sigma, St. Louis, MO), followed by Cy2-conjugated streptavidin (1:1,000, PA-42000; Amersham). Selected sections from all double-labeling techniques were incubated for 5 min with a 0.00125 μg/ml solution of 4,6-diamino-2-phenylindole (DAPI) in a Tris buffer.

Cell Counting and Confocal Analysis

Digital images of DAB-reacted coronal sections processed for caspase-8, caspase-9, and p53 were captured with the ×10 objective of a Nikon E-800 microscope attached to a Nikon Digital Eclipse DXM1200 camera and analyzed for positive cell counting. Cells were counted in three to five images per section in three or four sections for each of the three to five animals for each labeling, treatment, and survival time. Data are shown as mean ± SEM. In addition, double-immunofluorescent sections were analyzed for colocalization using a Leica TCS SP2 AOBS confocal microscope, and semi-quantitative analysis of the amount of double-labeled cells was carried out with the ×20 objective of a Nikon E-800 microscope in a minimum of three sections per animal for each double labeling and survival time.

Statistical Analysis

In total, 10 saline-injected animals, 30 NMDA-injected animals, and 20 intact control animals were used. Statistical analysis was carried out in Statview software. Significance among the five postlesion time intervals was determined by one-way analysis of variance (ANOVA) with Fisher's post hoc test, and $P < 0.05$ was considered significant.

RESULTS

Cortical injection of NMDA into the right sensorimotor cortex of 9-day-old rats caused neuronal degeneration accompanied by a glial response in the cortex at the level of the injection site and surrounding tissue extending to the corpus callosum, hippocampus, and dorsal striatum (Acarin et al., 1996, 1999a,b). Saline injection resulted in a mild glial response restricted to the needle track and slight expression of proapoptotic molecules but no neuronal death (Acarin et al., 2007).

Activation of the Apoptotic Intrinsic Pathway Through Caspase-9

In noninjected control animals, no caspase-9 expression was seen at any time point analyzed. Still, caspase-9 was observed in a few cells in the cortex of saline-injected control animals. In NMDA-lesioned animals caspase-9 activation was mainly seen in the cortex, hippocampus, and striatum. Slight expression was also seen in the corpus callosum. Cortical caspase-9 activation was seen predominantly at 10 hr postlesion (hpl) in the lesion core and in the adjacent cingulate cortex, and slight expression was also observed in caudal layer VI (Fig. 1A–C). In the hippocampus, caspase-9 activation was also seen only at 10 hpl and aws restricted to the cornu ammonis (CA) field, mainly CA1 (Fig. 1A,D,E). Caspase-9 was found showing granular intracellular staining (Fig. 1C), homogeneously distributed in the soma (Fig. 1F), and in the nucleus (Fig. 2C,F, I). F1 F2

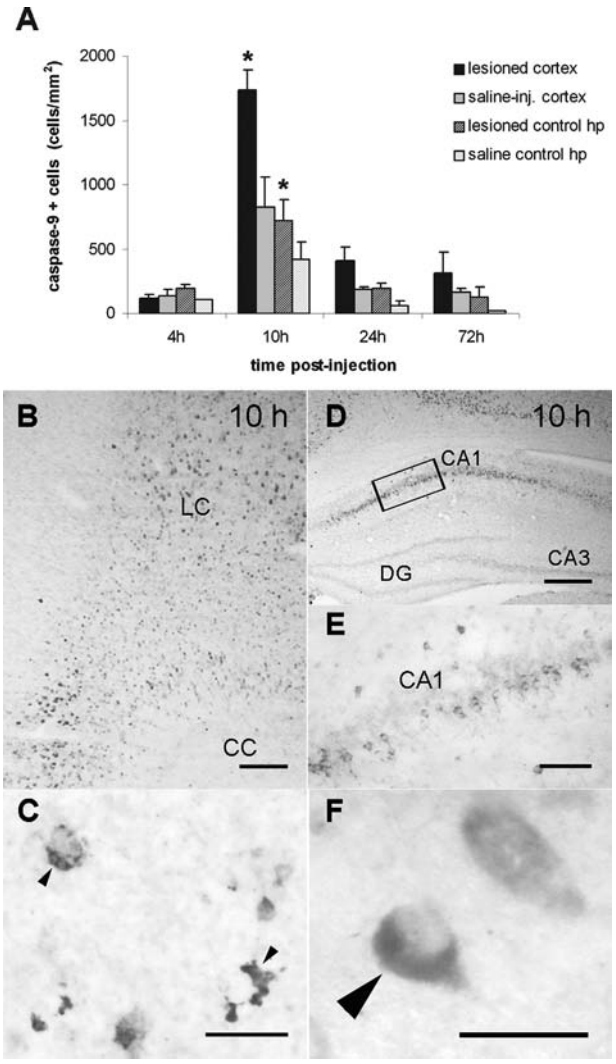


Fig. 1. Temporal and spatial pattern of caspase-9 activation in the cortex and hippocampus of excitotoxically damaged immature brain. **A:** Quantification of active caspase-9^+ cells in cortex and in hippocampus (hp) of saline-injected controls and NMDA-lesioned animals. A significant increase in active caspase-9^+ cells was seen in the hippocampal region and the lesioned cortex at 10 hpl (*$P < 0.05$ in relation to saline-injected). **B:** Active caspase-9 positive cells were located in the lesion core (LC) of the damaged cortex with a few also in the corpus callosum (CC) at 10 hpl. **C:** Active caspase-9 was associated with intracellular granular staining (arrowheads). **D:** Caspase-9 expression was also found in the cornu ammonis (CA1) of the ipsilateral hippocampus at 10 hpl. **E:** High magnification of boxed area in D. **F:** In the hippocampus, active caspase-9 was observed in the neuronal soma (arrowhead). Scale bars = 50 μm in B,D; 20 μm in C,E,F. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ID: haquek Date: 13/7/07 Time: 13:26 Path: J:/Production/JNR#/VOL00000/070254/3B2/C2JNR#070254

J_ID: Z3P Customer A_ID: 21450 Cadmus Art: JNR21450 Date: 13-JULY-07 Stage: I Page: 4

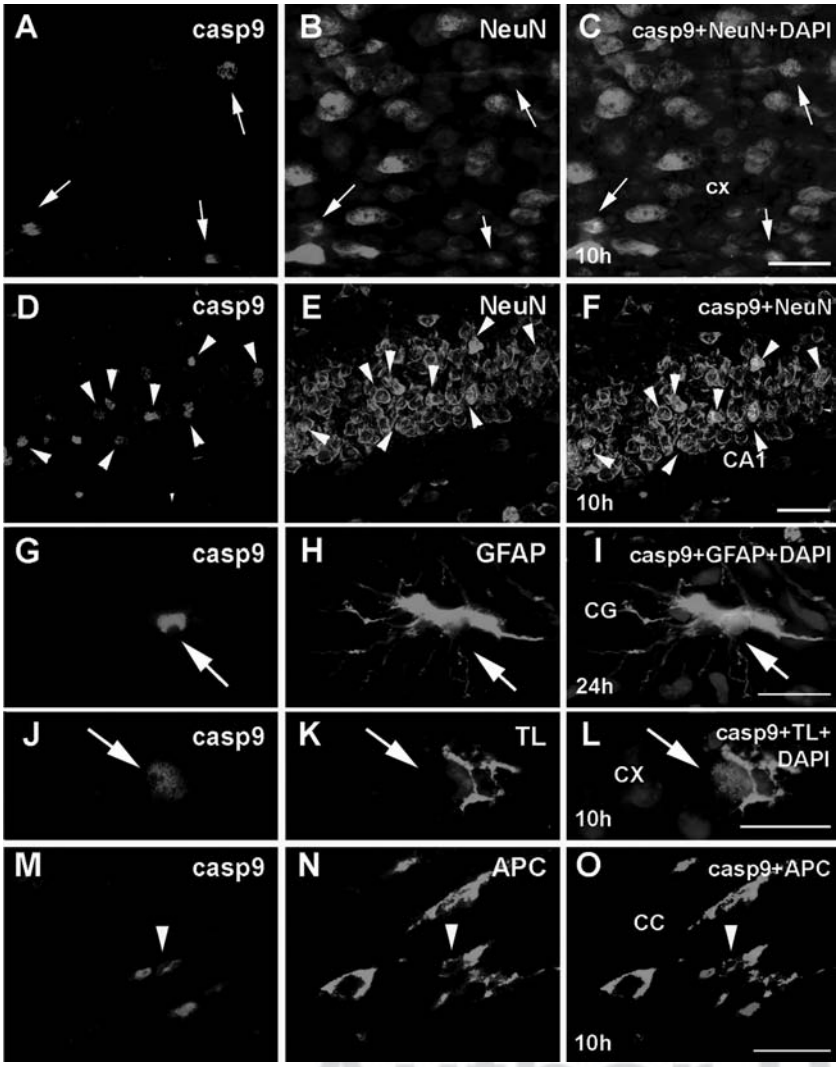


Fig. 2. Active caspase-9 (casp9) in neurons (NeuN), astrocytes (GFAP), microglia (TL) and oligodendrocytes (APC). Active caspase-9 was mainly found in neurons of the cortical lesion core (arrows in **A–C**) and in the hippocampal CA1 (arrowheads in **D–F**). In the cingulum, caspase-9 was seen in astrocytes (arrows in **G–I**). Microglia/macrophages were often seen surrounding caspase-9^+ cells (arrows in **J–L**). Some oligodendrocytes in the corpus callosum also showed active caspase-9 (arrowheads in **M–O**). Caspase-9 is shown in red; specific cell markers, NeuN, GFAP, TL, and APC, are shown in green; DAPI nuclear staining is shown in blue. CA1, cornu ammonis region 1; CC, corpus callosum; CX, cortex; CG, cingulum. Scale bars = 50 μm in C (applies to A–C); 50 μm in F (applies to D–F); 20 μm in I (applies to G–I); 20 μm in L (applies to J–L); 50 μm in O (applies to M–O). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Cellular distribution of caspase-9 was analyzed using double immunohistochemistry for active caspase-9 and specific markers for neurons (NeuN), astrocytes (GFAP), oligodendrocytes (APC), and microglia (TL; Fig. 2, Table I). Caspase-9 activation was most frequently seen in neuronal cells of the cortical lesion core (Fig. 2A–C) and in the hippocampus (Fig. 2D–F) at 10 hpl. In addition, caspase-9 was also seen in some GFAP$^+$ astrocytes located within the lesion core and in the adjacent white matter (Fig. 2G–I, Table I). Microglial cells did not generally show caspase-9 activation but were frequently seen in association with caspase-9^+ cells in the lesion core (Fig. 2J–L) as well as in scattered oligodendrocytes in the corpus callosum (Fig. 2M–O, Table I).

Activation of the Apoptotic Extrinsic Pathway Through Caspase-8

In noninjected control animals, no caspase-8 expression was seen at any postnatal age analyzed. In saline-injected control animals, caspase-8 was present only in a few cells restricted to the needle track, but expression never extended to the cortical parenchyma. In contrast, in NMDA-lesioned animals, caspase-8 immunolabeling was observed mainly in the lesioned cortex (Fig. 3A–D). In addition, scattered cells were also seen in hippocampus, dorsal striatum, and corpus callosum (Fig. 3E). In the lesioned cortex, overall caspase-8 immunolabeling significantly increased from 10 hpl, showed peak expression at 24 hpl, and decreased from 72 hpl. No differences were seen at 7 days postlesion (dpl; Fig. 3A). The time course of cortical caspase-8, however, was highly dependent on layer specificity (Fig. 3B). Layers I–III showed a significant increase in caspase-8-expressing cells from 10 hpl and peak expression at 24 hpl (Fig. 3B–D,F), which decreased by 72 hpl (Fig. 3B). In layers IV and V, expression was not as pronounced, and the appearance of caspase-8^+ cells was slightly delayed, increased at 10 hpl, and peaked at 24–72 hpl (Fig. 3B,C). In layer VI, caspase-8 activation

ID: haquek Date: 13/7/07 Time: 13:26 Path: J:/Production/JNR#/VOL00000/070254/3B2/C2JNR#070254

J_ID: Z3P Customer A_ID: 21450 Cadmus Art: JNR21450 Date: 13-JULY-07 Stage: I Page: 5

TABLE I. Semiquantitative Analysis of Neuronal, Astroglial, Microglial and Oligodendroglial Labeling for Caspase-9, Caspase-8, and p53 at Different Survival Times Following Excitotoxicity in the Immature Rat Brain*

		Caspase-9			Caspase-8			p53		
Time (hr)	Cell type	LC	VI/CC	HP	LC	VI/CC	HP	LC	VI/CC	HP
4 (n = 6)	Neurons	+	−/+	−	−/+	−	+	−	−/+	−
	Astrocytes	−	−/+	−	−	−/+	−/+	−	−/+	−
	Microglia	−/+	−/+	−	−	−	−	−	+	−
	Oligodendrocytes	−	−	−	−	−	−	−	−	−
10 (n = 6)	Neurons	+++	+	++	+++	+	+	++	+	−
	Astrocytes	+	++	+	+	++	−/+	+	+	−/+
	Microglia	−/+	−/+	−	+	−	−	+	++	−
	Oligodendrocytes	−	++	−	−	−/+	−	−	−	−
24 (n = 6)	Neurons	++	+	−/+	++	++	+	+++	+	+
	Astrocytes	+	++	−	+	++	−/+	+	++	+
	Microglia	−	−	−	++	++	−	+	++	−
	Oligodendrocytes	−	−/+	−	−/+	+	−	−	−	−
72 (n = 6)	Neurons	−/+	+	−/+	+	+++	++	+	++	+
	Astrocytes	−/+	−/+	−	+	++	+	−/+	++	−
	Microglia	+	−/+	−	+	−/+	−	+	+	−
	Oligodendrocytes	−	−/+	−	−/+	−	−	−	−	−

*LC, lesion core; VI/CC, cortical layer VI/corpus callosum; HP, hippocampus; −, no labeled cells; +/−, very occasional labeled cells; +, less than half the cells were labeled; ++, more than half the cells were labeled; and +++, most cells were labeled.

was further delayed, showing some positive cells at 10 hpl and the maximal number of caspase-8^+ cells at 72 hpl (Fig. 3B,H,I). In all cortical layers, caspase-8 immunostaining was predominantly perinuclear and sometimes located in the nucleus of cortical cells (Fig. 3F,I). In the corpus callosum, however, immunolabeling was usually seen in the cytoplasm (Fig. 3E,G).

Cell-specific expression of caspase-8, analyzed by double immunohistochemistry using specific markers, showed that the majority of caspase-8^+ cells in the different cortical layers were neurons ($NeuN^+$; Fig. 4A–C), especially in layers I–V at 10–24 hpl and in layer VI at 72 hpl (Table I). Additionally, caspase-8 labeling was also found in the nucleus of reactive $GFAP^+$ astrocytes (Fig. 4D–F, Table I) mostly located in layer VI and the corpus callosum. Microglial cells showing nuclear caspase-8 immunoreactivity were also seen in the lesion core and layer VI, mainly at 24 hpl (Fig. 4G–I, Table I). Finally, occasional caspase-8 labeling was also seen in oligodendrocytes in the cingulum and corpus callosum (Fig. 4J–L, Table I).

Correspondence Between Caspase-9/Caspase-8 and Caspase-3 Activation

To determine whether caspase-3 activation resulted from the intrinsic pathway (by caspase-9 activation) or the extrinsic pathway (by caspase-8 activation), double immunofluorescence for active caspase-3 and either active caspase-9 or caspase-8 was performed (Fig. 5). As previously reported (Acarin et al., 2007), in the excitotoxically lesioned postnatal brain, caspase-3 activation was significantly increased from 10 to 72 hpl and showed peak values at 24 hpl, in correspondence with the time course of caspase-8 labeling but delayed in relation to caspase-9 activation. In general, in the lesioned cortex, density of caspase-3^+ cells was always higher than that of caspase-9^+ or caspase-8^+ cells (Fig. 5A–M). However, at 4 and 10 hpl, the ratio between density of caspase-9^+ cells and caspase-3^+ cells showed maximal values of 0.78 and 1.02, respectively. Notably, correlation between caspase-3 and caspase-9 was strongly diminished from 24 hpl, when maximal caspase-3 activation was found. In contrast, the ratio between density of caspase-3^+ cells and caspase-8^+ cells was uniform from 4 to 72 hpl, showing ratio values between 0.45 and 0.29.

Expression of p53

Noninjected and saline-injected control animals showed slight expression of p53. In contrast, NMDA-lesioned animals showed changes in p53 expression mainly in the ipsilateral cortex and the corpus callosum, but mild hippocampal expression was also seen. Cortical p53 expression was significantly increased from 10 to 72 hpl (Fig. 6A), with peak expression at 24 hpl (Fig. 6A,B), and down-regulation to control values at 7 dpl (data not shown). In the corpus callosum, the density of $p53^+$ cells was slightly lower but showed a similar time course (Fig. 6A,B). Both in the cortex (Fig. 6C) and in the corpus callosum (Fig. 6D), labeling was usually cytoplasmatic. The analysis of double immunostaining for specific cellular markers showed that p53 was expressed mainly in neurons located within the cortical lesion core (Fig. 7A–C, Table I) and in astrocytes of cortical layer VI and the adjacent corpus callosum (Fig. 7D–F, Table I). However, some astrocytes in the lesion core and in the hippocampus were also $p53^+$ (data not shown). Some microglial cells also showed p53 expression (Fig. 7G–I, Table I). No p53 expression was detected in oligodendrocytes. Notably, colocalization between p53 and active caspase-3 was only occasionally found (Fig. 7J–L).

ID: haquek Date: 13/7/07 Time: 13:26 Path: J:/Production/JNR#/VOL00000/070254/3B2/C2JNR#070254

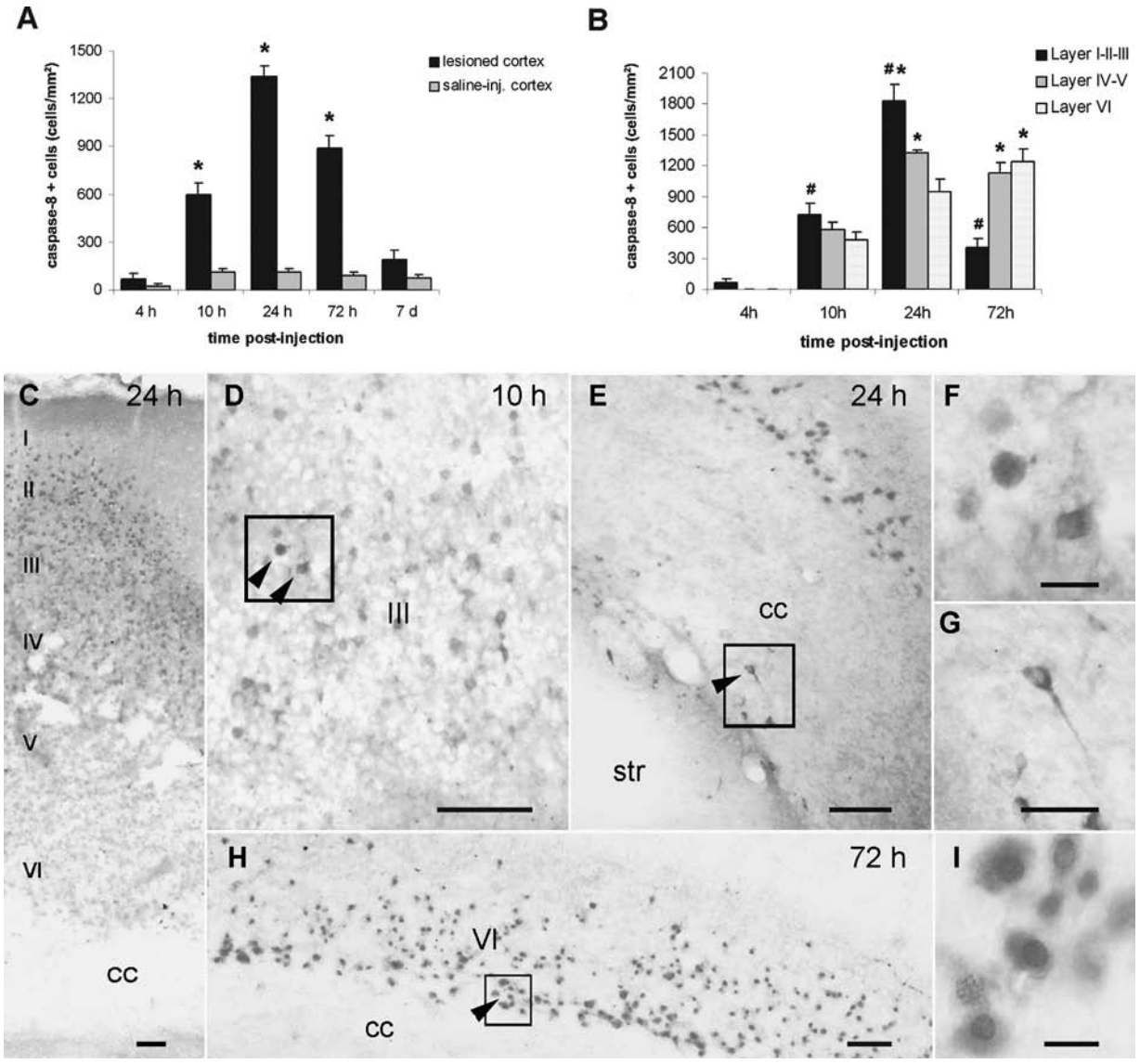


Fig. 3. Temporal pattern of caspase-8 expression in distinct regions of the damaged immature brain. **A:** Quantification of caspase-8 expression in the damaged cortex showed a significant increase ($^{\star}P < 0.05$ in comparison with saline controls) from 10 hpl to 24 hpl, when peak expression was seen, and then decreased, but was still significant by 72 hpl. **B:** Temporal caspase-8 expression was layer specific in the cortex at the different survival times postinjury. There were significant differences in the number of caspase-8^{+} cells in layers I–III compared with layers IV/V and layer VI at 24 and 72 hpl ($^{\#}P < 0.05$). Cell density in layers I–III was significantly higher at 10–24 hpl and lower at 72 hpl. Maximal number of caspase-8^{+} cells was seen at 24 hpl in layers I–III ($^{\star}P < 0.05$ in comparison with other time points), at 24–72 hpl in layers IV/V ($^{\star}P < 0.05$), and at 72 hpl in layer VI ($^{\star}P < 0.05$). **C:** Differential caspase-8 expression in the damaged cortical layers. Caspase-8 was seen mainly in layers I–IV at 24 hpl. **D:** Localization of caspase-8 in the nucleus of some cells (arrowheads) at 10 hpl in cortical layer III. High magnification is shown in **F**. **E:** Scattered caspase-8^{+} cells (arrowhead) in the corpus callosum at 24 hpl. High magnification showing cytoplasmic labeling is shown in **G**. No labeling was seen in the striatum (str). **H:** Caspase-8 expression (arrowhead) in cortical layer VI at 72 hpl. **I:** High magnification shows nuclear and cytoplasmatic labeling. Scale bars = 50 μm in C–E,H; 20 μm in F,G,I. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

This study shows that, after postnatal excitotoxicity, neurons in the cortical lesion core activate the intrinsic pathway mainly through caspase-9, but also the extrinsic pathway through caspase-8 and the p53-dependent pathway. Activation of these pathways is maintained until 24 hpl, when maximal neuronal caspase-3 activation and apoptosis have been shown to occur in this lesion model (Acarin et al., 2007). In astrocytes, the previously reported widespread activation of caspase-3 in the absence of cell death (Acarin et al., 2007) is shown here generally not to correlate with activation of upstream

J_ID: Z3P Customer A_ID: 21450 Cadmus Art: JNR21450 Date: 13-JULY-07 Stage: I Page: 7

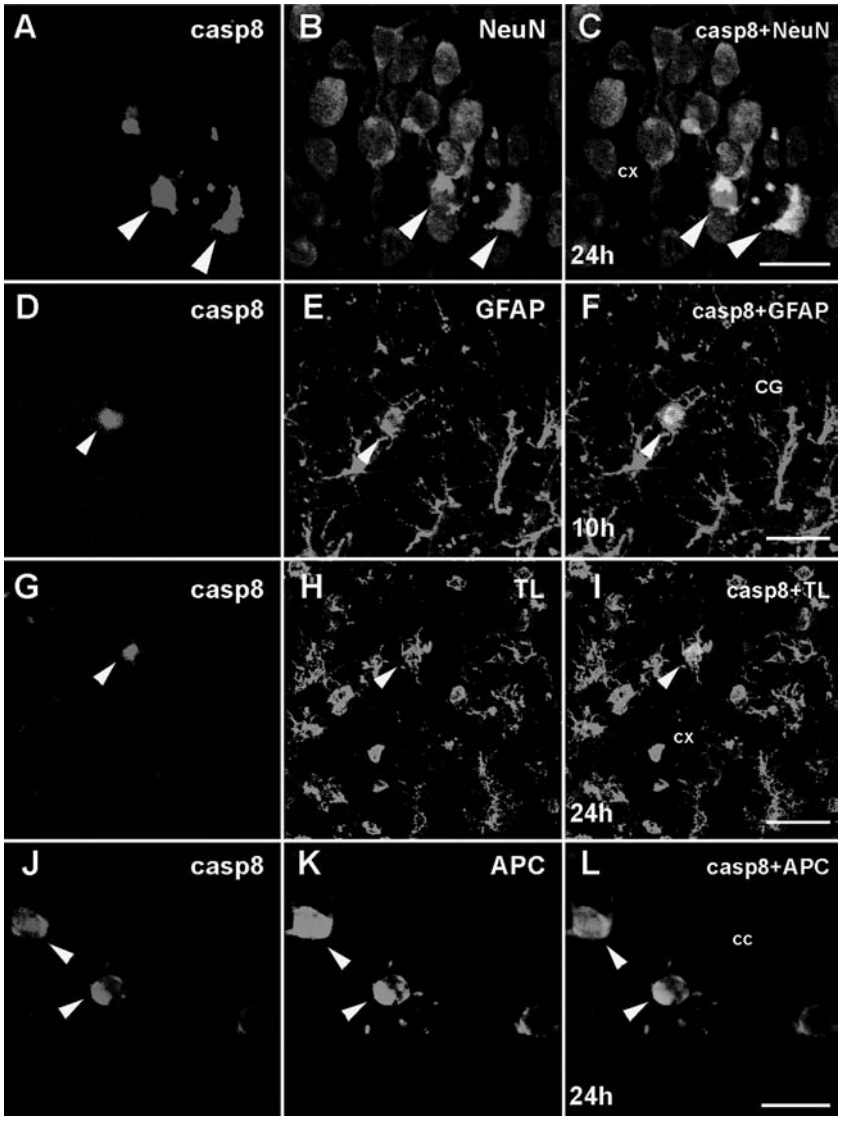


Fig. 4. Caspase-8 (casp8) in neurons (NeuN), astrocytes (GFAP), microglia (TL), and oligodendrocytes (APC). Most caspase-8-positive cells were neurons as seen in the cortex at 24 hpl (arrowheads in **A–C**). Caspase-8 was also found in some astrocytes (arrowhead in **D–F**) located in layer VI and the corpus callosum. Caspase-8 was also found in some microglia/macrophages within the lesion core (arrowhead in **G–I**). Oligodendrocytes in the corpus callosum were also found to express caspase-8 (arrowheads in **J–L**). Caspase-8 is shown in red; specific cell markers NeuN, GFAP, APC, and TL are shown in green. CC, corpus callosum; CG, cingulum; CX, cortex. Scale bars = 20 μm in C (applies to A–C); 20 μm in F (applies to D–F); 20 μm in I (applies to G–I); 20 μm in L (applies to J–L). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

pathways mediated by caspase-8, caspase-9, and p53. Activation of these pathways is only relevant in astrocytes of cortical layer VI and the adjacent corpus callosum.

Activation of Caspase Pathways in Neuronal Cells

We show here an important participation of the mitochondrial pathway in cortical and hippocampal neurons within the first 10 hpl, when maximal caspase-9 activation colocalized with caspase-3 in these cells, preceding previously reported caspase-3 and $TUNEL^+$ maximal cell density at 24 hpl (Acarin et al., 2007). As shown in the present report, caspase-9/caspase-3 double-labeled neurons frequently displayed apoptotic features and cytoplasmic vacuolation. Excitotoxicity by overestimulation of glutamate receptors induces an increase in reactive oxygen species that alters mitochondrial respiration and cytochrome c release and activates the intrinsic apoptotic pathway (Mattson, 2003). In this sense, it is thought that neuronal cells largely use the intrinsic pathway to undergo apoptotic cell death, not only after excitotoxicity (Henshall et al., 2001) but also in other injury paradigms, such as focal and global ischemia (Benchoua et al., 2001; Cao et al., 2002; Cho et al., 2003; Ferrer et al., 2003; Love, 2003; Ding et al., 2004) and traumatic brain injury (Yakovlev and Faden, 2001; Knoblach et al., 2002), in adult animals. In the immature brain, in agreement with our study, neuronal caspase-9 activation occurs within the first 24 hr after hypoxia-ischemia and traumatic brain damage (Felderhoff-Mueser et al., 2002; Khurana et al., 2002; Benjelloun et al., 2003; Hagberg, 2004) and caspase-9 blockade induces some degree of neuroprotection after hypoxia-ischemia (Feng et al., 2003b).

In addition to caspase-9, damaged cortical neurons show a reduced and somewhat delayed activation of the

ID: haquek Date: 13/7/07 Time: 13:27 Path: J:/Production/JNR#/VOL00000/070254/3B2/C2JNR#070254

J_ID: Z3P Customer A_ID: 21450 Cadmus Art: JNR21450 Date: 13-JULY-07 Stage: I Page: 8

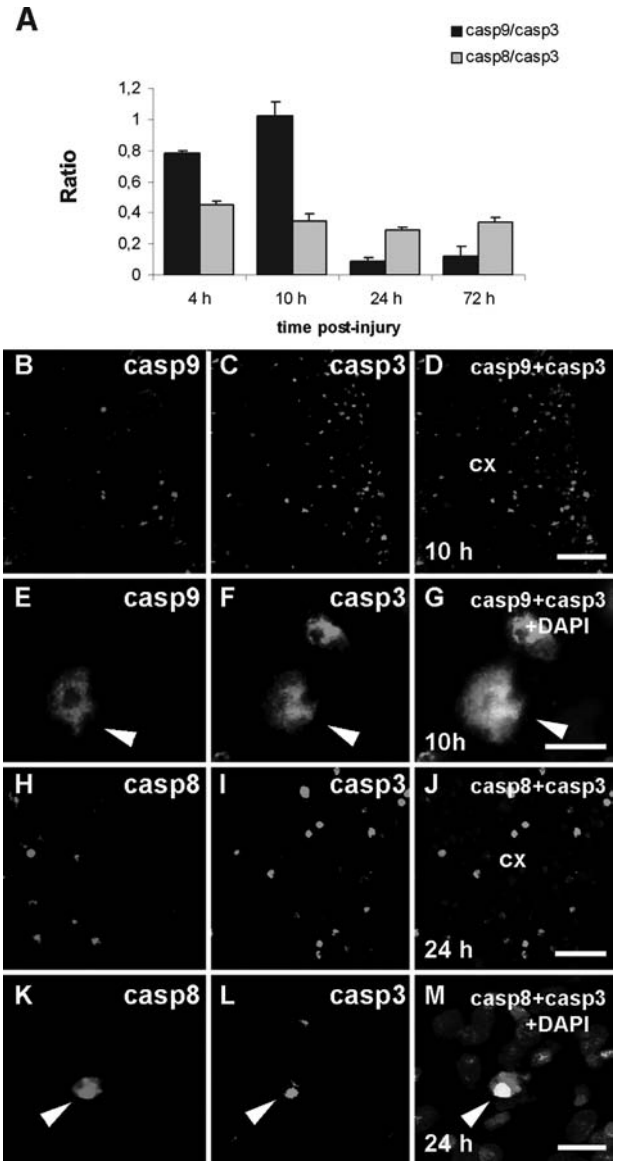


Fig. 5. Colocalization of caspase-8 and caspase-9 with caspase-3 in the lesioned cortex at different survival times postlesion. **A:** Mean ratio values for caspase-8/caspase-3 and caspase-9/caspase-3 at 4, 10, 24, and 72 hpl. Highest correlation is seen at 10 hpl in the ratio of caspase-9/caspase-3 double-labeled cells. The caspase-8/caspase-3 ratio is uniform and always lower than 0.5. Representative images of caspase-9/caspase-3 double labeling in the lesioned cortex at 10 hpl (**B–G**) and caspase-8/caspase-3 labeling at 24 hpl (**H–M**). The majority of caspase-9^+ cells colocalized with caspase-3 (B–G) and showed condensed nuclei, as seen with DAPI nuclear staining (G). Lower amounts of caspase-8^+ cells colocalized with caspase-3 (H–J). Caspase-8 and -9 are shown in red; caspase-3 is shown in green; DAPI nuclear staining is shown in blue. cx, Cortex. Scale bars = 50 μm in D (applies to B–D); 50 μm in G (applies to E–G); 50 μm in J (applies to H–J); 50 μm in M (applies to K–M). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

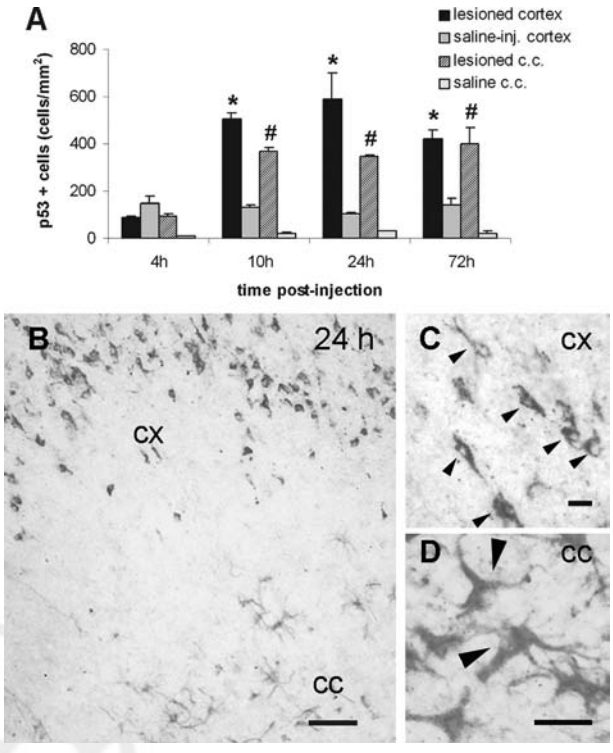


Fig. 6. Temporal pattern of p53 expression in the cortex and corpus callosum after immature brain injury. **A:** Quantification of cells expressing p53. There was a significant increase in $p53^+$ cells at all times from 10 hpl to 72 hpl in the cortex ($^{\star}P < 0.05$) and corpus callosum (cc; $^{\#}P < 0.05$) compared with saline-injected controls animals. **B:** p53 Expression was seen in cells in the damaged cortex and cingulum of the cc. Higher magnification images of p53-expressing cells in the cortex (arrowheads in **C**) and the cingulum of the corpus callosum, where cytoplasmic labeling was seen in cells resembling astrocytes (arrowheads in **D**). CC, corpus callosum, CX, cortex. Scale bars = 50 mm in B; 20 μm in C,D. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

extrinsic pathway through caspase-8 (peak values at 24 hpl), which can also contribute to caspase-3 activation and cell death (Schulze-Osthoff et al., 1998; Thorburn, 2004), according to several lines of evidence suggesting that the death receptor pathway participates in the neuronal death after excitotoxicity, ischemia, and trauma, in both the adult and the immature brain (Velier et al., 1999; Krupinski et al., 2000; Northington et al., 2001a,b; Beer et al., 2001; Benchoua et al., 2001; Qiu et al., 2002; Felderhoff-Mueser et al., 2002; Feng et al., 2003a; Li et al., 2006). In this regard, neurons express the death receptors FAS and TNFR1 (Rosenbaum et al., 2000; Northington et al., 2001a; Qiu et al., 2002; Felderhoff-Mueser et al., 2002; Ferrer et al., 2003; Henshall et al., 2003), which correlates with injury-induced neuronal and glial expression of their ligands FASL and TNFα, respectively (Choi and Benveniste, 2004; Swanson et al., 2004; Planas et al., 2006). Particularly after postnatal excitotoxicity, we have previously shown TNFα production at 10–24 hpl by neurons and astrocytes located in the lesion border and in cortical layer VI (Acarin et al., 2000a), the same area where we find delayed up-regulation of caspase-8. Interestingly, during

ID: haquek Date: 13/7/07 Time: 13:27 Path: J:/Production/JNR#/VOL00000/070254/3B2/C2JNR#070254

J_ID: Z3P Customer A_ID: 21450 Cadmus Art: JNR21450 Date: 13-JULY-07 Stage: I Page: 9

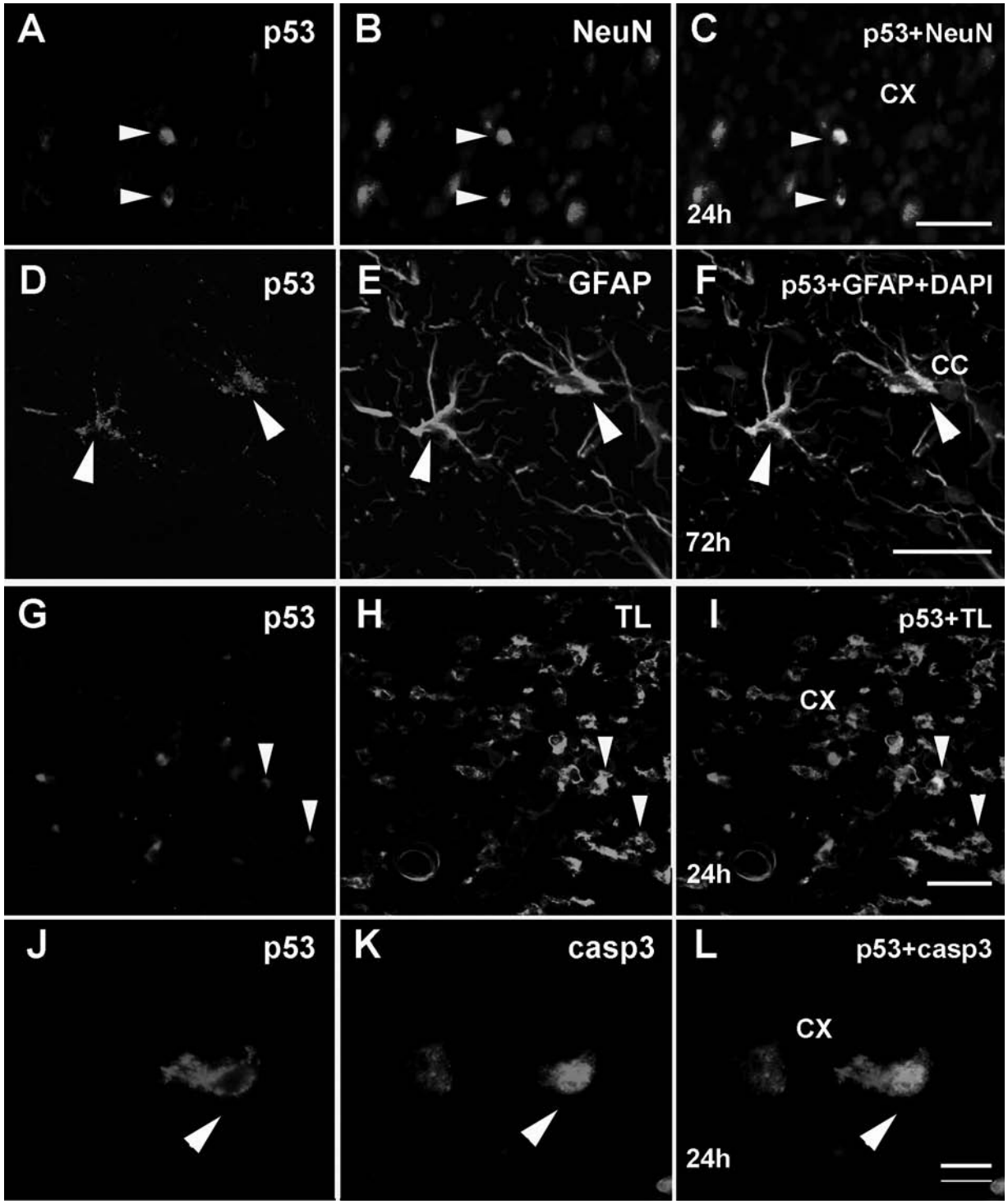


Fig. 7. p53 Expression in neurons (NeuN), astrocytes (GFAP), and microglia (TL) and correlation with caspase-3 (casp3). p53 Expression was seen in neurons in the cortical lesion core (**A–C**; arrowheads), and in some astrocytes of layer VI and the corpus callosum (**D–F**; arrowheads). $p53^+$ Microglia/macrophages were present in the lesioned cortex (**G–I**; arrowheads). Only scattered $p53^+$ cells colocalized with caspase-3 (**J–L**). p53 Is shown in red; specific cell markers NeuN, GFAP, and TL and caspase-3 are shown in green; DAPI nuclear staining is shown in blue. CC, corpus callosum; CX, cortex. Scale bars = 50 μm in C (applies to A–C); 50 μm in F (applies to D–F); 50 μm in I (applies to G–I); 50 μm in L (applies to J–L). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ID: haquek Date: 13/7/07 Time: 13:27 Path: J:/Production/JNR#/VOL00000/070254/3B2/C2JNR#070254

early postnatal life and in this area, neurons are either layer VI or remnants of subplate neurons, a transient population of cells highly vulnerable to excitotoxicity and hypoxia/ischemia (Ghosh et al., 1990; Ghosh and Shatz, 1993; Allendoerfer and Shatz, 1994; McQuillen et al., 2003; McQuillen and Ferriero, 2005). Their depletion leads to failure of thalamocortical innervation or abnormal thalamocortical connections, depending on the age when the insult occurs (Ghosh et al., 1990; Ghosh and Shatz, 1993; McQuillen and Ferriero, 2005).

Caspase-3 can also be activated by the p53 pathway, essentially after excitotoxicity (Sakhi et al., 1994), where oxidative stress occurs (Acarin et al., 2002, 2005). Although we have observed p53 expression in damaged neurons, there was little correlation between p53 and caspase-3. Insofar as p53-induced caspase-3 activation is mediated either through the intrinsic mitochondrial pathway by inducing Bax expression (Culmsee and Mattson, 2005) or through the extrinsic pathway by activation of DR5 receptor and caspase-8 (Finnberg and El-Deiry, 2004), it is likely that the time-frame between p53 expression and caspase-3 activation may be too long to find both molecules at the same time in the same cell.

Glial Activation of Caspase Pathways in Layer VI and Adjacent White Matter

Widespread astroglial activation of caspase-3 (Acarin et al., 2007) does not correlate with activation of upstream caspases-8 and -9, the main initiators of caspase-dependent apoptotic cascades. This finding, together with the fact that new roles for caspase-3 have been reported (Schwerk and Schulze-Osthoff, 2003; McLaughlin, 2004), leads to the suggestion that other signaling mechanisms involved in astroglial cell activation might also cleave caspase-3.

The differential expression of caspase proteins in glial cells located in layer VI and adjacent white matter deserves discussion. Caspase-9, caspase-8, and p53 were found in layer VI and corpus callosum astrocytes, where astroglial cells show increased GFAP and cell hypertrophy and display nuclear active caspase-3 in the absence of cell death (Acarin et al., 1999b, 2007). Similarly, following hypoxia-ischemia in postnatal day 7 rats, activation of the intrinsic pathway in astrocytes has been reported in the penumbra, an area of delayed neurodegeneration, but not in the lesion core (Benjelloun et al., 2003). To our knowledge, no description of caspase-9 activation in astrocytes has been reported after adult brain damage. In contrast, astroglial activation of caspase-8 occurs after adult brain trauma and in vitro paradigms (Beer et al., 2001; Wosik et al., 2001; Falsig et al., 2004), but this is the first study reporting caspase-8 in astrocytes after immature brain injury. It should be noted that caspase-8 immunoreactivity may be interpreted either as caspase-8 activation or as expression of procaspase-8, in that the antibody used has been shown to recognize both the active form and the 55-kDa proform of this caspase after immature brain damage (Northington et al., 2001a). However, the presence of active caspase-8 and/or changes in procaspase-8 expression demonstrates the involvement of the extrinsic pathway and upstream receptor activation. In this regard, after in vitro and in vivo stimuli, astrocytes express FASR and TNFR1 on their surface (Botchkina et al., 1997; Choi and Benveniste, 2004; Yin et al., 2004; Kuno et al., 2006). However, astrocytes are known generally to be resistant to FAS-mediated cell death (Choi et al., 1999; Lee et al., 2000), and astroglial FAS activation is related to cytokine and chemokine release, including interleukin-6 (IL-6), IL-8, and monocyte chemoattractant protein-1 (MCP-1) through nuclear factor-κB (NFκB) activation (Choi and Benveniste, 2004). Similarly, activation of astroglial TNFR1 receptors mediates production of MCP-1, cell adhesion molecules such as ICAM-1, and neurotrophic factors (Yin et al., 2004; Kuno et al., 2006). Therefore, caspase-8 in astrocytes may constitute a step in the FASR and/or TNFR1 astroglial inflammatory response, insofar as it is localized in the cell nuclei and correlates with activation of NFκB (Acarin et al., 2000b) and production of TNFα and IL-6 (Acarin et al., 2000a) in layer VI and corpus callosum reactive astrocytes. This correspondence has been demonstrated in lymphocytes, where caspase-8 signaling induces activation of NFκB, and whether caspase-8 mediates inflammatory or apoptotic cell death depends, at least in part, on activation levels of this caspase (Alam et al., 1999; Lamkanfi et al., 2007; Lemmers et al., 2007).

Finally, astrocytes also express p53 in this region, as has previously been demonstrated after ischemia (Chung et al., 2002). Besides its participation in oxidative stress-mediated cell death of astrocytes (Yung et al., 2004), and in correlation with the absence of astroglial death that we have previously described (Acarin et al., 2007), p53 has also been implicated in cell cycle exit and differentiation in astroglial cell lines in vitro (Kokunai et al., 1998).

With regard to microglial cells, in the present work, we show that only significant expression of caspase-8 and p53 was seen in layer VI and corpus callosum in the excitotoxically damaged postnatal brain. This occurs in the absence of microglial caspase-3 activation in this model (Acarin et al., 2007). The lack of caspase-9 activation in microglia agrees with other studies of immature and adult brain damage (Krupinski et al., 2000; Benchoua et al., 2001; Henshall et al., 2001; Knoblach et al., 2002; Benjelloun et al., 2003). Notably, caspase-9 activation is implicated in the monocytic differentiation to macrophages in response to macrophage colony-stimulating factor (M-CSF), where cleaving of caspase-9 precedes caspase-3 activation and subsequent cleavage of acinus protein in the absence of cell death (Sordet et al., 2002). It should be noted that activation of M-CSF receptors is a well-established mechanism of microglial activation, mediating proliferation, cytokine release, and phagocytosis (Imai and Kohsaka, 2002), and the lack of caspase-9/caspase-3 activation in these cells could imply differential signaling cascades of this receptor

J_ID: Z3P Customer A_ID: 21450 Cadmus Art: JNR21450 Date: 13-JULY-07 Stage: I Page: 11

in peripheral monocytes vs. endogenous microglial cells. Finally, only significant activation of caspase-9 was seen in oligodendrocytes at 10 hpl, although this occurs in the absence of caspase-3 activation or TUNEL staining in this cell type, as previously described (Acarin et al., 2007).

In conclusion, after postnatal excitotoxicity, well-known activators of caspase-3, mainly the mitochondrial intrinsic pathway, mediate neuronal caspase-3 activation associated with apoptotic cell death. However, in astroglial cells, where caspase-3 activation is not generally related to cell death but to cell adaptation mechanisms such as cytoskeletal changes (Acarin et al., 2007), cleavage of caspase-3 is not preceded by activation of intrinsic and extrinsic pathways. Further studies are needed to elucidate putative alternative mechanisms of caspase-3 activation in glial cells.

ACKNOWLEDGMENTS

We thank M. Martil for his technical help and M. Roldan from Servei Microscopia (UAB) for helpful assistance with confocal microscopy. S.V. and M.F. hold an FI fellowship from the Autonomous University of Barcelona.

REFERENCES

Acarin L, Gonzalez B, Castellano B, Castro AJ. 1996. Microglial response to N-methyl-D-aspartate-mediated excitotoxicity in the immature rat brain. J Comp Neurol 367:361–374.

Acarin L, González B, Castro AJ, Castellano B. 1999a. Primary cortical glial reaction vs. secondary thalamic glial response in the excitotoxically injured young brain. Microglial/macrophage response and MHC class I and II expression. Neuroscience 89:549–565.

Acarin L, González B, Hidalgo J, Castro AJ, Castellano B. 1999b. Primary cortical glial reaction vs. secondary thalamic glial response in the excitotoxically injured young brain. Astroglial response and metallothionein expression. Neuroscience 92:827–839.

Acarin L, Gonzalez B, Castellano B. 2000a. Neuronal, astroglial and microglial cytokine expression after an excitotoxic lesion in the immature rat brain. Eur J Neurosci 12:3505–3520.

Acarin L, Gonzalez B, Castellano B. 2000b. STAT3 and NFkappaB activation precedes glial reactivity in the excitotoxically injured young cortex but not in the corresponding distal thalamic nuclei. J Neuropathol Exp Neurol 59:151–163.

Acarin L, Peluffo H, Gonzalez B, Castellano B. 2002. Expression of inducible nitric oxide synthase and cyclooxygenase-2 after excitotoxic damage to the immature rat brain. J Neurosci Res 68:745–754.

Acarin L, Peluffo H, Barbeito L, Castellano B, Gonzalez B. 2005. Astroglial nitration after postnatal excitotoxic damage: correlation with nitric oxide sources, cytoskeletal, apoptotic and antioxidant proteins. J Neurotrauma 22:189–200.

Acarin L, Villapol S, Faiz M, Rohn TT, Castellano B, Gonzalez B. 2007. Caspase-3 activation in astrocytes following postnatal excitotoxic damage correlates with cytoskeletal remodeling but not with cell death or proliferation. Glia (in press).

Alam A, Cohen LY, Aouad S, Sekaly RP. 1999. Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells. J Exp Med 190:1879–1890.

Allendoerfer KL, Shatz CJ. 1994. The subplate, a transient neocortical structure: its role in the development of connections between thalamus and cortex. Annu Rev Neurosci 17:185–218.

Beer R, Franz G, Krajewski S, Pike BR, Hayes RL, Reed JC, Wang KK, Klimmer C, Schmutzhard E, Poewe W, Kampfl A. 2001. Temporal and spatial profile of caspase 8 expression and proteolysis after experimental traumatic brain injury. J Neurochem 78:862–873.

Benchoua A, Guegan C, Couriaud C, Hosseini H, Sampaio N, Morin D, Onteniente B. 2001. Specific caspase pathways are activated in the two stages of cerebral infarction. J Neurosci 21:7127–7134.

Benjelloun N, Joly LM, Palmier B, Plotkine M, Charriaut-Marlangue C. 2003. Apoptotic mitochondrial pathway in neurones and astrocytes after neonatal hypoxia-ischaemia in the rat brain. Neuropathol Appl Neurobiol 29:350–360.

Botchkina GI, Meistrell ME, 3rd, Botchkina IL, Tracey KJ. 1997. Expression of TNF and TNF receptors (p55 and p75) in the rat brain after focal cerebral ischemia. Mol Med 3:765–781.

Cao G, Luo Y, Nagayama T, Pei W, Stetler RA, Graham SH, Chen J. 2002. Cloning and characterization of rat caspase-9: implications for a role in mediating caspase-3 activation and hippocampal cell death after transient cerebral ischemia. J Cereb Blood Flow Metab 22:534–546.

Cho S, Liu D, Gonzales C, Zaleska MM, Wood A. 2003. Temporal assessment of caspase activation in experimental models of focal and global ischemia. Brain Res 982:146–155.

Choi C, Benveniste EN. 2004. Fas ligand/Fas system in the brain: regulator of immune and apoptotic responses. Brain Res Brain Res Rev 44:65–81.

Choi C, Park JY, Lee J, Lim JH, Shin EC, Ahn YS, Kim CH, Kim SJ, Kim JD, Choi IS, Choi IH. 1999. Fas ligand and Fas are expressed constitutively in human astrocytes and the expression increases with IL-1, IL-6, TNF-alpha, or IFN-gamma. J Immunol 162:1889–1895.

Chung YH, Shin CM, Kim MJ, Lee EY, Kim G, Cha CI. 2002. Enhanced expression of p53 in reactive astrocytes following transient focal ischemia. Neurol Res 24:324–328.

Costantini P, Bruey JM, Castedo M, Metivier D, Loeffler M, Susin SA, Ravagnan L, Zamzami N, Garrido C, Kroemer G. 2002. Pre-processed caspase-9 contained in mitochondria participates in apoptosis. Cell Death Differ 9:82–88.

Culmsee C, Mattson MP. 2005. p53 in neuronal apoptosis. Biochem Biophys Res Commun 331:761–777.

Didier M, Bursztajn S, Adamec E, Passani L, Nixon RA, Coyle JT, Wei JY, Berman SA. 1996. DNA strand breaks induced by sustained glutamate excitotoxicity in primary neuronal cultures. J Neurosci 16:2238–2250.

Ding C, He Q, Li PA. 2004. Activation of cell death pathway after a brief period of global ischemia in diabetic and non-diabetic animals. Exp Neurol 188:421–429.

Eizenberg O, Faber-Elman A, Gottlieb E, Oren M, Rotter V, Schwartz M. 1996. p53 Plays a regulatory role in differentiation and apoptosis of central nervous system-associated cells. Mol Cell Biol 16:5178–5185.

Falsig J, Latta M, Leist M. 2004. Defined inflammatory states in astrocyte cultures: correlation with susceptibility towards CD95-driven apoptosis. J Neurochem 88:181–193.

Felderhoff-Mueser U, Sifringer M, Pesditschek S, Kuckuck H, Moysich A, Bittigau P, Ikonomidou C. 2002. Pathways leading to apoptotic neurodegeneration following trauma to the developing rat brain. Neurobiol Dis 11:231–245.

Feng Y, Fratkin JD, LeBlanc MH. 2003a. Inhibiting caspase-8 after injury reduces hypoxic-ischemic brain injury in the newborn rat. Eur J Pharmacol 481:169–173.

Feng Y, Fratkin JD, LeBlanc MH. 2003b. Inhibiting caspase-9 after injury reduces hypoxic ischemic neuronal injury in the cortex in the newborn rat. Neurosci Lett 344:201–204.

ID: haquek Date: 13/7/07 Time: 13:27 Path: J:/Production/JNR#/VOL00000/070254/3B2/C2JNR#070254

Ferrer I, Friguls B, Dalfo E, Justicia C, Planas AM. 2003. Caspase-dependent and caspase-independent signalling of apoptosis in the penumbra following middle cerebral artery occlusion in the adult rat. Neuropathol Appl Neurobiol 29:472–481.

Finnberg N, El-Deiry WS. 2004. Activating FOXO3a, NF-kappaB and p53 by targeting IKKs: an effective multi-faceted targeting of the tumor-cell phenotype? Cancer Biol Ther 3:614–616.

Fischer U, Janicke RU, Schulze-Osthoff K. 2003. Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ 10:76–100.

Fulda S, Debatin KM. 2004. Signaling through death receptors in cancer therapy. Curr Opin Pharmacol 4:327–332.

Ghosh A, Shatz CJ. 1993. A role for subplate neurons in the patterning of connections from thalamus to neocortex. Development 117:1031–1047.

Ghosh A, Antonini A, McConnell SK, Shatz CJ. 1990. Requirement for subplate neurons in the formation of thalamocortical connections. Nature 347:179–181.

Hagberg H. 2004. Mitochondrial impairment in the developing brain after hypoxia-ischemia. J Bioenerg Biomembr 36:369–373.

Hengartner MO. 2000. The biochemistry of apoptosis. Nature 407:770–776.

Henshall DC, Bonislawski DP, Skradski SL, Araki T, Lan JQ, Schindler CK, Meller R, Simon RP. 2001. Formation of the Apaf-1/cytochrome c complex precedes activation of caspase-9 during seizure-induced neuronal death. Cell Death Differ 8:1169–1181.

Henshall DC, Araki T, Schindler CK, Shinoda S, Lan JQ, Simon RP. 2003. Expression of death-associated protein kinase and recruitment to the tumor necrosis factor signaling pathway following brief seizures. J Neurochem 86:1260–1270.

Imai Y, Kohsaka S. 2002. Intracellular signaling in M-CSF-induced microglia activation: role of Iba1. Glia 40:164–174.

Khurana P, Ashraf QM, Mishra OP, Delivoria-Papadopoulos M. 2002. Effect of hypoxia on caspase-3, -8, and -9 activity and expression in the cerebral cortex of newborn piglets. Neurochem Res 27: 931–938.

Knoblach SM, Nikolaeva M, Huang X, Fan L, Krajewski S, Reed JC, Faden AI. 2002. Multiple caspases are activated after traumatic brain injury: evidence for involvement in functional outcome. J Neurotrauma 19:1155–1170.

Kokunai T, Izawa I, Tamaki N. 1998. Overexpression of p21WAF1/CIP1 induces cell differentiation and growth inhibition in a human glioma cell line. Int J Cancer 75:643–648.

Krupinski J, Lopez E, Marti E, Ferrer I. 2000. Expression of caspases and their substrates in the rat model of focal cerebral ischemia. Neurobiol Dis 7:332–342.

Kumar S. 2007. Caspase function in programmed cell death. Cell Death Differ 14:32–43.

Kuno R, Yoshida Y, Nitta A, Nabeshima T, Wang J, Sonobe Y, Kawanokuchi J, Takeuchi H, Mizuno T, Suzumura A. 2006. The role of TNF-alpha and its receptors in the production of NGF and GDNF by astrocytes. Brain Res 1116:12–18.

Lamkanfi M, Festjens N, Declercq W, Vanden Berghe T, Vandenabeele P. 2007. Caspases in cell survival, proliferation and differentiation. Cell Death Differ 14:44–55.

Lane DP. 1993. Cancer. A death in the life of p53. Nature 362:786–787.

Lee SJ, Zhou T, Choi C, Wang Z, Benveniste EN. 2000. Differential regulation and function of Fas expression on glial cells. J Immunol 164:1277–1285.

Lemmers B, Salmena L, Bidere N, Su H, Matysiak-Zablocki E, Murakami K, Ohashi P, Jurisicova A, Lenardo M, Hakem R, Hakem A. 2007. Essential role for caspase-8 in toll-like receptors and NFkappa B signalling. J Biol Chem 282:7416–7423.

Li T, Lu C, Xia Z, Xiao B, Luo Y. 2006. Inhibition of caspase-8 attenuates neuronal death induced by limbic seizures in a cytochrome c-dependent and Smac/DIABLO-independent way. Brain Res 1098:204–211.

Love S. 2003. Apoptosis and brain ischaemia. Prog Neuropsychopharmacol Biol Psychiatry 27:267–282.

Marks N, Berg MJ. 1999. Recent advances on neuronal caspases in development and neurodegeneration. Neurochem Int 35:195–220.

Mattson MP. 2003. Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. Neuromol Med 3:65–94.

McLaughlin B. 2004. The kinder side of killer proteases: caspase activation contributes to neuroprotection and CNS remodeling. Apoptosis 9:111–121.

McQuillen PS, Ferriero DM. 2005. Perinatal subplate neuron injury: implications for cortical development and plasticity. Brain Pathol 15:250–260.

McQuillen PS, Sheldon RA, Shatz CJ, Ferriero DM. 2003. Selective vulnerability of subplate neurons after early neonatal hypoxia-ischemia. J Neurosci 23:3308–3315.

Northington FJ, Ferriero DM, Flock DL, Martin LJ. 2001a. Delayed neurodegeneration in neonatal rat thalamus after hypoxia-ischemia is apoptosis. J Neurosci 21:1931–1938.

Northington FJ, Ferriero DM, Martin LJ. 2001b. Neurodegeneration in the thalamus following neonatal hypoxia-ischemia is programmed cell death. Dev Neurosci 23:186–191.

Planas AM, Gorina R, Chamorro A. 2006. Signalling pathways mediating inflammatory responses in brain ischaemia. Biochem Soc Trans 34:1267–1270.

Qiu J, Whalen MJ, Lowenstein P, Fiskum G, Fahy B, Darwish R, Aarabi B, Yuan J, Moskowitz MA. 2002. Upregulation of the Fas receptor death-inducing signaling complex after traumatic brain injury in mice and humans. J Neurosci 22:3504–3511.

Renolleau S, Benjelloun N, Ben-Ari Y, Charriaut-Marlangue C. 1997. Regulation of apoptosis-associated proteins in cell death following transient focal ischemia in rat pups. Apoptosis 2:368–376.

Rosenbaum DM, Gupta G, D'Amore J, Singh M, Weidenheim K, Zhang H, Kessler JA. 2000. Fas (CD95/APO-1) plays a role in the pathophysiology of focal cerebral ischemia. J Neurosci Res 61:686–692.

Sakhi S, Bruce A, Sun N, Tocco G, Baudry M, Schreiber SS. 1994. p53 Induction is associated with neuronal damage in the central nervous system. Proc Natl Acad Sci U S A 91:7525–7529.

Sakhi S, Sun N, Wing LL, Mehta P, Schreiber SS. 1996. Nuclear accumulation of p53 protein following kainic acid-induced seizures. Neuroreport 7:493–496.

Salvesen GS, Dixit VM. 1999. Caspase activation: the induced-proximity model. Proc Natl Acad Sci U S A 96:10964–10967.

Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME. 1998. Apoptosis signaling by death receptors. Eur J Biochem 254:439–459.

Schwerk C, Schulze-Osthoff K. 2003. Non-apoptotic functions of caspases in cellular proliferation and differentiation. Biochem Pharmacol 66:1453–1458.

Sordet O, Rebe C, Plenchette S, Zermati Y, Hermine O, Vainchenker W, Garrido C, Solary E, Dubrez-Daloz L. 2002. Specific involvement of caspases in the differentiation of monocytes into macrophages. Blood 100:4446–4453.

Stefanis L. 2005. Caspase-dependent and -independent neuronal death: two distinct pathways to neuronal injury. Neuroscientist 11:50–62.

Strasser A, O'Connor L, Dixit VM. 2000. Apoptosis signaling. Annu Rev Biochem 69:217–245.

Swanson RA, Ying W, Kauppinen TM. 2004. Astrocyte influences on ischemic neuronal death. Curr Mol Med 4:193–205.

Thorburn A. 2004. Death receptor-induced cell killing. Cell Signal 16:139–144.

Velier JJ, Ellison JA, Kikly KK, Spera PA, Barone FC, Feuerstein GZ. 1999. Caspase-8 and caspase-3 are expressed by different populations of cortical neurons undergoing delayed cell death after focal stroke in the rat. J Neurosci 19:5932–5941.

Wosik K, Becher B, Ezman A, Nalbantoglu J, Antel JP. 2001. Caspase 8 expression and signaling in Fas injury-resistant human fetal astrocytes. Glia 33:217–224.

Yakovlev AG, Faden AI. 2001. Caspase-dependent apoptotic pathways in CNS injury. Mol Neurobiol 24:131–144.

Yin L, Ohtaki H, Nakamachi T, Kudo Y, Makino R, Shioda S. 2004. Delayed expressed TNFR1 co-localize with ICAM-1 in astrocyte in mice brain after transient focal ischemia. Neurosci Lett 370:30–35.

Yung HW, Bal-Price AK, Brown GC, Tolkovsky AM. 2004. Nitric oxide-induced cell death of cerebrocortical murine astrocytes is mediated through p53- and Bax-dependent pathways. J Neurochem 89: 812–821.

AQ1: Please give sources for these grants.

ARTICLE III

"*Survivin and Heat Shock Protein 25/27 colocalize with cleaved caspase-3 in surviving reactive astrocytes following excitotoxicity to the immature brain*"

Submitted (2007).

Survivin and Heat Shock Protein 25/27 colocalize with cleaved caspase-3 in surviving reactive astrocytes following excitotoxicity to the immature brain

ABSTRACT

Following immature excitotoxic brain damage, distinct patterns of caspase activation have been described in neurons and glial cells. Neuronal cells show activation of the mitochondrial pathway, caspase-3 cleavage and apoptotic cell death, while reactive astrocytes show caspase-3 clevage that is not always correlated with enzymatic protease activity and does not generally terminate in cell death. Accordingly, the aim of the present study was to evaluate the astrocytic colocalization of cleaved caspase-3 and several anti-apoptotic proteins of the inhibitor of apoptosis proteins family (IAPs), such as survivin and cIAP-2, and the Heat Shock Proteins (HSPs) family, HSP25/27 and HSC70/HSP70, which can all prevent caspases from cleaving their substrates. At several survival times ranging from 4 hours to 14 days after cortical excitotoxic damage induced by N-methyl-D-aspartate (NMDA) injection at postnatal day 9 in rat pups, single and double immunohistochemical techniques were performed in free floating cryostat sections and sections were analyzed by confocal microscopy. Our results show that survivin and HPS25/27 are mainly expressed in reactive astrocytes of the damaged cortex and the adjacent white matter. In addition, both molecules strongly colocalize with cleaved caspase-3. Survivin is mainly located in the nucleus, like cleaved caspase-3; while HSP25/27 is cytoplasmatic but very frequently found in cells showing nuclear caspase-3. cIAP-2 was mostly found in damaged neurons but also in glial scar reactive astrocytes and showed certain correlation with caspase-3. Hsc70/Hsp70 was only expressed in injured neurons and did not correlate with caspase-3. Thus, we conclude that mainly survivin and HSP25/27 may participate in the inhibition of cleaved caspase-3 in reactive astrocytes and may be involved in protecting astrocytes after injury.

Survivin and Heat Shock Protein 25/27 colocalize with cleaved caspase-3 in surviving reactive astrocytes following excitotoxicity to the immature brain

Sonia Villapol*, Laia Acarin*, Maryam Faiz, Bernardo Castellano and Berta Gonzalez
Medical Histology. Department of Cell Biology, Physiology and Immunology. Institute of Neuroscience. Faculty of Medicine. Autonomous University of Barcelona, 08193. Spain.

KEY WORDS; Survivin, IAP, HSP, neonatal, caspase-3, excitotoxicity

ABSTRACT

Following immature excitotoxic brain damage, distinct patterns of caspase activation have been described in neurons and glial cells. Neuronal cells show activation of the mitochondrial pathway, caspase-3 cleavage and apoptotic cell death, while reactive astrocytes show caspase-3 clavage that is not always correlated with enzymatic protease activity and does not generally terminate in cell death. Accordingly, the aim of the present study was to evaluate the astrocytic colocalization of cleaved caspase-3 and several anti-apoptotic proteins of the inhibitor of apoptosis proteins family (IAPs), such as survivin and cIAP-2, and the Heat Shock Proteins (HSPs) family, HSP25/27 and HSC70/HSP70, which can all prevent caspases from cleaving their substrates. At several survival times ranging from 4 hours to 14 days after cortical excitotoxic damage induced by N-methyl-D-aspartate (NMDA) injection at postnatal day 9 in rat pups, single and double immunohistochemical techniques were performed in free floating cryostat sections and sections were analyzed by confocal microscopy. Our results show that survivin and HPS25/27 are mainly expressed in reactive astrocytes of the damaged cortex and the adjacent white matter. In addition, both molecules strongly colocalize with cleaved caspase-3. Survivin is mainly located in the nucleus, like cleaved caspase-3; while HSP25/27 is cytoplasmatic but very frequently found in cells showing nuclear caspase-3. cIAP-2 was mostly found in damaged neurons but also in glial scar reactive astrocytes and showed certain correlation with caspase-3. HSC70/HSP70 was only expressed in injured neurons and did not correlate with caspase-3. Thus, we conclude that mainly survivin and HSP25/27 may participate in the inhibition of cleaved caspase-3 in reactive astrocytes and may be involved in protecting astrocytes after injury.

INTRODUCTION

Acute damage to the immature and adult brain induces neuronal activation of caspase cascades that culminate in apoptotic cell death. We have recently described that excitotoxic damage to the immature rat brain results in activation of the mitochondrial pathway through caspase-9 in neuronal cells, and correlates with activation of the executioner caspase-3 and neuronal apoptotic cell death in different brain regions (Acarin et al., 2007; Villapol et al., 2007). In addition, cleavage of caspase-3 is also observed in astroglial cells within hours after the damage and until glial scar formation one week later. At early survival times, astroglial cleavage of caspase-3 correlates with enzymatic activity of caspase-3 and the presence of caspase-mediated cleavage of their main cytoskeletal protein, glial fibrillary acidic protein (GFAP) (Acarin et al., 2007; Villapol et al., 2007). However, from one day post-lesion, cleaved caspase-3 is still present in astrocytes but no enzymatic caspase-3 activity is detected and very little astroglial cell death occurs (Acarin et al., 2007). Studies of apoptotic cascades in peripheral tissues (see (McLaughlin, 2004) for review) show that cleaved caspase-3 may be present without causing significant substrate proteolysis of cellular components and inducing subsequent apoptosis. These studies suggest that powerful intrinsic cell signaling mechanisms exist which limit executioner caspases protease activity. Accordingly, several families of proteins such as the Inhibitors of Apoptosis (IAP) and the heat shock proteins (HSP) have been shown to block the activity of cleaved caspase-3, both in the nervous system and in peripheral organs. IAPs are a family of pro-survival proteins that act downstream of caspase cleavage to directly bind and inhibit the activity of caspase-9 and the executor caspases -3 and -7, halting the apoptotic process. Their activity is based on the presence of two general types of baculoviral IAP repeat (BIR) domains: Type I domains which most commonly bind and inhibit caspases, and Type II domains which can bind caspases but can also act on the cell cycle. Four of the existing mammalian IAPs, including cIAP-1, cIAP-2, XIAP, and NAIP, contain three type I BIR domains. The other four IAPs, named livin, APOLLON/BRUCE, ts-IAP/ML-IAP and survivin contain only one BIR domain or type II domains, and can therefore participate in cell cycle regulation besides their role in caspase inhibition (Clem et al., 1996). After damage to the CNS, several IAPs including XIAP, cIAP-1, NIAP and survivin have been shown to provide neuroprotection against ischemia and trauma, mainly through caspase-3 inhibition in neuronal cells (see (Robertson et al., 2000; Ferrer and Planas, 2003) for review). In addition, survivin is expressed in reactive astrocytes after adult traumatic injury, where it has been shown to participate in cellular proliferation and it has been shown to colocalize with caspase-3 (Johnson et al., 2004; Johnson et al., 2005). HSPs are molecular chaperones involved in the regulation of cellular homeostasis and cell survival (for review (Sreedhar and Csermely, 2004)). Under normal conditions, HSPs are essential for the folding of nascent proteins, protein translocation and maintenance of multi-protein complexes. After stress or cell damage, they prevent formation of protein aggregates and either contribute to the re-folding of denatured and misfolded proteins or target them for destruction by the proteasome (Sreedhar and Csermely, 2004).

Grant sponsor: This work was supported by BFU2005-02783 (Ministry of Science), Marato TV3 Foundation 061710 and 2005SGR-00956 (AGAUR). SV and MF held a FI fellowship from the Autonomous University of Barcelona.
*Corresponding Authors to: Laia Acarin and Sonia Villapol. Medical Histology, Torre M5. Departament Cell Biology, Physiology and Immunology and Institute of Neurosciences Autonomous University of Barcelona. Bellaterra 08193, Barcelona, Spain. Phone: +34-935811826. Fax. +34-935812392
E-mail address: laia.acarin@uab.es, sonia.villapol@uab.es.

HSPs are a multi-protein family that range in molecular size from 10 to 110 kD and are divided into HSP10, HSP25/27, HSP40, HSP60, HSP70, HSP90 and HSP110 subfamilies with distinct functions within the cells. HSC70/HSP70 is present in two forms in the brain, the constitutively expressed HSC70 and the inducible form of the protein, named HSP70 (Rubio et al., 2002; Giffard et al., 2004). HSP70 is able to prevent the formation of the apoptosome by binding to Apaf-1 and blocking the activation of caspase-9 and caspase-3, and inhibiting the appearance of apoptosis after DNA damage (Matsumori et al., 2005). After acute brain damage, such as ischemia, trauma, and oxidative injury, overexpression of HSP70 protects neuronal cells against injury (Sharp et al., 1999; Giffard et al., 2004; Yenari et al., 2005). The family of low molecular weight HSP, including HSP25/27, stabilizes the cytoskeleton and the proteolytic cleavage of actin and reduces oxidative DNA damage by sequestering cytochrome c and procaspase-3 (Concannon et al., 2003). We and other laboratories have described the upregulation of small heat shock proteins of the HSP25/27 family in reactive astrocytes following immature (Sanz et al., 2001; Acarin et al., 2002) and adult brain damage (Kato et al., 1995; Plumier et al., 1996; Anguelova and Smirnova, 2000; Currie et al., 2000; Nishino and Nowak, 2004).

According to this, the aim of the present study was to determine whether following excitotoxic immature brain damage, the IAPs, survivin and cIAP-2, and the HSPs, HSP70 and HSP25/27, colocalize with active caspase-3 in reactive astrocytes showing cleavage of this executor caspase in the absence of cell death.

MATERIALS AND METHODS

Excitotoxic lesions and tissue processing

Neonatal Long Evans black-hooded rat pups of both genders were used on postnatal day 9 (P9) (Janvier, France). They were anesthetized with isofluorane (Baxter International Inc) and received an intracerebral injection of 20 nmol of N-methyl-D-aspartate (NMDA) (Sigma-Aldrich, St. Louis, MO, USA) diluted in 0.15 µl of saline solution (0.9% NaCl) at a rate of 0.05 µl/min using an automatic injector. Saline-control animals received an injection of 0.15 µl of the saline solution as vehicle. All intracerebral injections were made into the right sensorimotor cortex at the level of the coronal suture (2 mm lateral of bregma and 0.5 mm depth) using a stereotaxic frame (Kopf Instruments). After 4, 10, 24 and 72 hours, and 7 and 14 days post-injection, rats were anesthetized and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4). Brains were removed and immersed in the same fixative for 4 hours at 4ºC and cryoprotected in 30% sucrose solution before being frozen with dry CO_2. Four NMDA-injected and 2 saline-injected animals were used for each of the five survival times. Our experimental animal work was conducted according to Spanish regulations in agreement with European Union directives and was approved by the ethical commission of the Autonomous University of Barcelona.

Single immunohistochemistry for survivin, cIAP-2, HSP25/27 and HSC70/HSP70

Coronal sections (30-µm-thick) were obtained using a Leitz cryostat. Free-floating parallel sections were processed for endogenous peroxidase inactivation and blocked in 0.05 M Tris-buffered saline (TBS) pH=7.4, containing 10% fetal calf serum and 1% triton X-100 for 1 hour. Afterwards, sections were incubated overnight at 4ºC with either one of the following primary antibodies: rabbit polyclonal antibody anti-cIAP-2 (1:400, AB3615, Chemicon); rabbit polyclonal antibody anti-survivin (1:2000, IMG-5754, Imgenex); rabbit polyclonal antibody anti-HSP25 (recognizing both HSP25 and HSP27, 1:400, SPA-801, Stressgen) or mouse monoclonal antibody anti-Hsc/Hsp70 (1:200, SPA-820, Stressgen). As negative controls, sections were incubated in media lacking the primary antibody. After several washes, sections were incubated for 1 hour with either biotinylated anti-rabbit secondary antibody (1:400, RPN1004V1, Amersham) or biotinylated anti-mouse secondary antibody (1:400, RPN1001V1, Amersham). Specific labeling was visualized by incubation with avidin-peroxidase (1:400, P0364, Dakopatts) for 1 hour and subsequent 3, 3´-diaminobenzidine (DAB)-hydrogen peroxide developing procedure. Sections were dehydrated through a graded series of ethanol, cleared in xylene and coverslipped.

Double immunofluorescence labeling for cIAP-2, survivin, HSP25/27 and HSC70/HSP70 with cell specific markers and caspase-3

For double labeling, sections were processed with antibodies against Hsc70/Hsp70, cIAP-2, survivin or Hsp25 as described above, but incubated with either secondary cy3-conjugated anti-mouse antibody (1:1000, PA43002V, Amersham) (for Hsc70/Hsp70) or secondary cy3-conjugated anti-rabbit antibody (1:1000, PA430024V, Amersham) (for survivin, Hsp25, and cIAP-2). After rinsing, sections were further incubated with either one of the following primary antibodies: polyclonal rabbit anti-cleaved caspase-3 (p17 or p19 subunit) (1:700, #9661, Cell Signaling) for caspase-3 labeling; polyclonal rabbit anti-Glial Fibrillary Acidic Protein (GFAP) (1:1000, Z-0224, Dakopatts), monoclonal mouse anti-GFAP (1:700, #3670, Cell Signaling), or monoclonal mouse anti-vimentin (1:1000, Chemicon, MO0725) for astroglial labeling; and monoclonal mouse anti-Neuronal Nuclear Antigen (NeuN) (1:500, MAB377, Chemicon) or monoclonal mouse anti-microtubule Associated Protein 2 (MAP2) (1:1000, Chemicon, MAB3418), for neuronal labeling. Immunostaining was visualized with either cy2-conjugated anti-rabbit secondary antibody (1:1000, PA-42004, Amersham) or cy2-conjugated anti-mouse secondary antibody (1:1000, PA-42004, Amersham). Selected sections of all double labeling techniques were incubated for 5 min with a 0.00125µg/ml solution of 4, 6-diamino-2-phenylindole (DAPI) in Tris buffer. Immediately after, sections were rinsed in TBS and TB, dehydrated and mounted and coverslipped.

Cell counting and statistical analysis

Digital images of DAB-reacted and double immunofluorescent coronal sections were captured with the 20X and 40X objective of a Nikon E-800 microscope attached to a Nikon Digital Eclipse DXM1200 color camera. For the quantification of double labeled cells, cells were counted in 3-5 images per section and brain region and in 3-4 sections for each of the 3 animals per labeling and survival time. Statistical analysis was carried out using Statview software. Statistical significance was determined by one-way analysis of variance (ANOVA), with Fisher's post hoc test and $p<0.05$. In addition, for colocalization studies, immunofluorescent labeled sections were analyzed using a LEICA TCS SP2 AOBS confocal microscope.

RESULTS

Cortical injection of the excitotoxin NMDA into the right hemisphere of postnatal day 9 rat pups caused a lesion involving neuronal loss and a glial response in the entire thickness of the cortex and surrounding areas, extending to the corpus callosum, dorsal striatum and rostral hippocampus, which has been previously described in detail (Acarin et al., 1999a; Acarin et al., 1999b; Acarin et al., 2001).

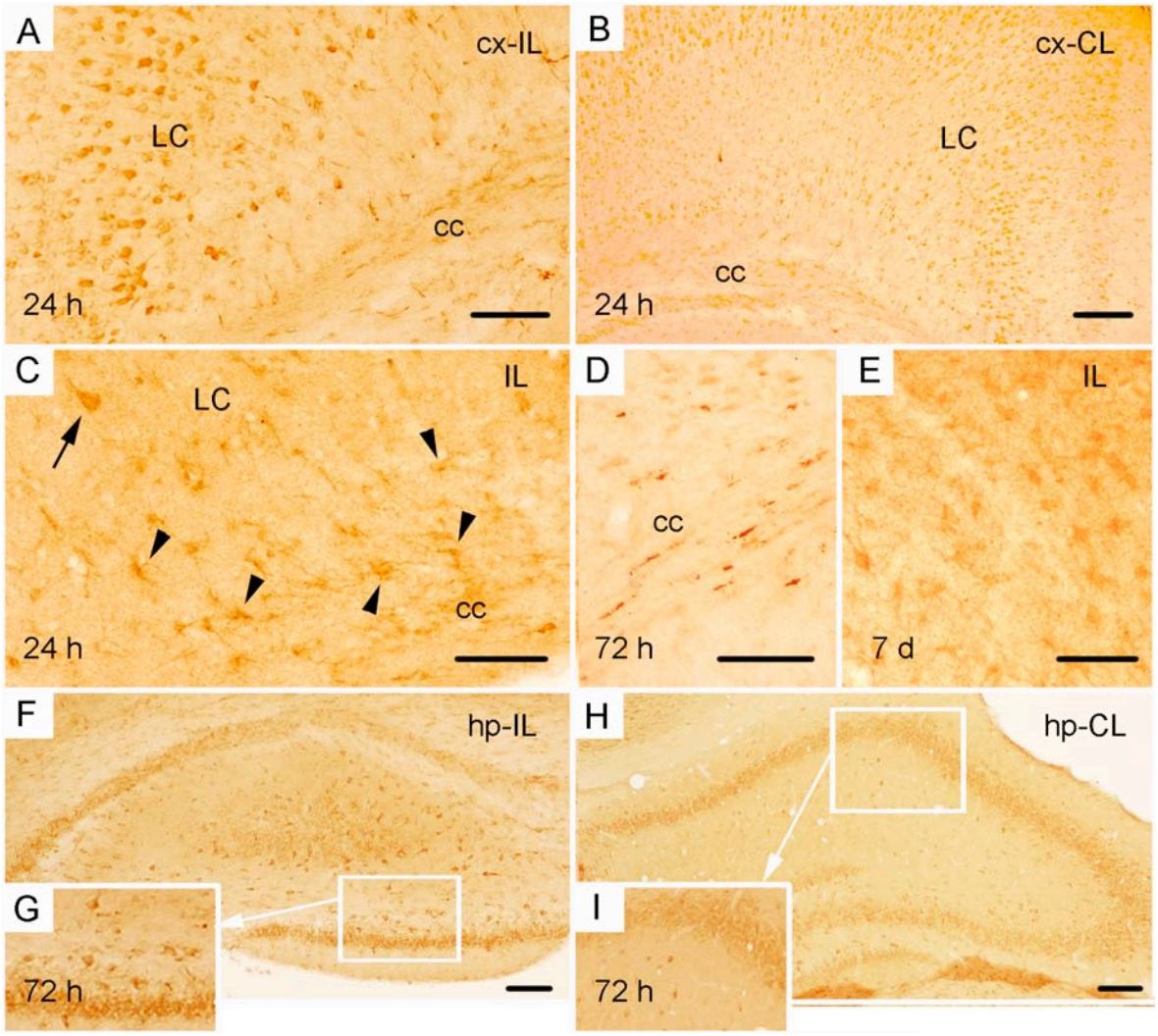


Figure 1. *Survivin expression in the damaged immature brain*. Intensive staining is seen in the ipsilateral cortical lesion core at 24 hpl (hours post-lesion) (A), although low survivin expression is also seen in the contralateral cortex (B). A high magnification showing survivin expression in the cytoplasm of star-shaped cells (arrowheads in C) and pyramidal cells (arrow in C) in the lesion core. At 72 hpl, survivin is localized in cell nuclei of the corpus callosum (D), and at 7 days post-lesion is confined to the glial scar (E). Neuronal survivin expression is also observed in the ipsilateral hippocampus (F, inset in G) at 72 hpl, and in the contralateral hippocampus to a lesser extent (H, inset in I). Abbreviations: cc (corpus callosum); CL (contralateral hemisphere); cx-CL (contralateral cortex); cx-IL (ipsilateral cortex); hp-CL (contralateral hippocampus); hp-IL (ipsilateral hippocampus); IL (ipsilateral hemisphere); LC (lesion core). Scale bars = 20 µm.

Excitotoxic damage induced activation of the mitochondrial apoptotic pathway through caspase-9 in neuronal cells followed by caspase-3 activation and TUNEL labeling (Acarin et al., 2007, Villapol 2007). However, in reactive astrocytes, caspase-3 activation was not correlated with cell death (Acarin et al., 2007) and persisted until two weeks post-lesion. Injection of a control saline solution resulted in mild tissue disruption restricted to the area of the needle track, with slight expression of pro-apoptotic molecules and a focal and transient glial response that lasted until 3 days post-injection.

Survivin expression is mainly found in the ipsilateral hemisphere but mild staining is also observed in contralateral brain regions

In the ipsilateral hemisphere of saline-injected control animals, survivin was observed in a few cells in cortical areas and hippocampus (data not shown). In NMDA-lesioned animals, survivin+ cells were seen mainly in the damaged cortex, corpus callosum and hippocampus in the ipsilateral hemisphere (IL) (Fig. 1A, C-F), but milder survivin+ cells were also found in corresponding areas of the contralateral hemisphere (CL) (Fig. 1B, H). In the IL cortex, at 10-24 hours post-lesion, survivin labeling was mainly nuclear and perinuclear (Fig. 1C) and found in the lesion core. From 24 hours, survivin expression increased and spread to the cortical lesion border and the corpus callosum, where labeling was mainly observed in the cytoplasm (Fig. 1C, D). At 72 h post-lesion, cortical survivin expression was found in the lesion border and broadening to cortical layer VI. By 7 days post-lesion staining was confined to layer VI and corpus callosum in both hemispheres, but restricted to the cortical glial scar in star-shaped cells in the IL (Fig. 1E). Additionally, survivin+ cells were also seen throughout the hippocampus (Fig. 1F,G), mainly in the IL hemisphere, although some labeling was seen in the CL hippocampus (Fig. 1H,I).

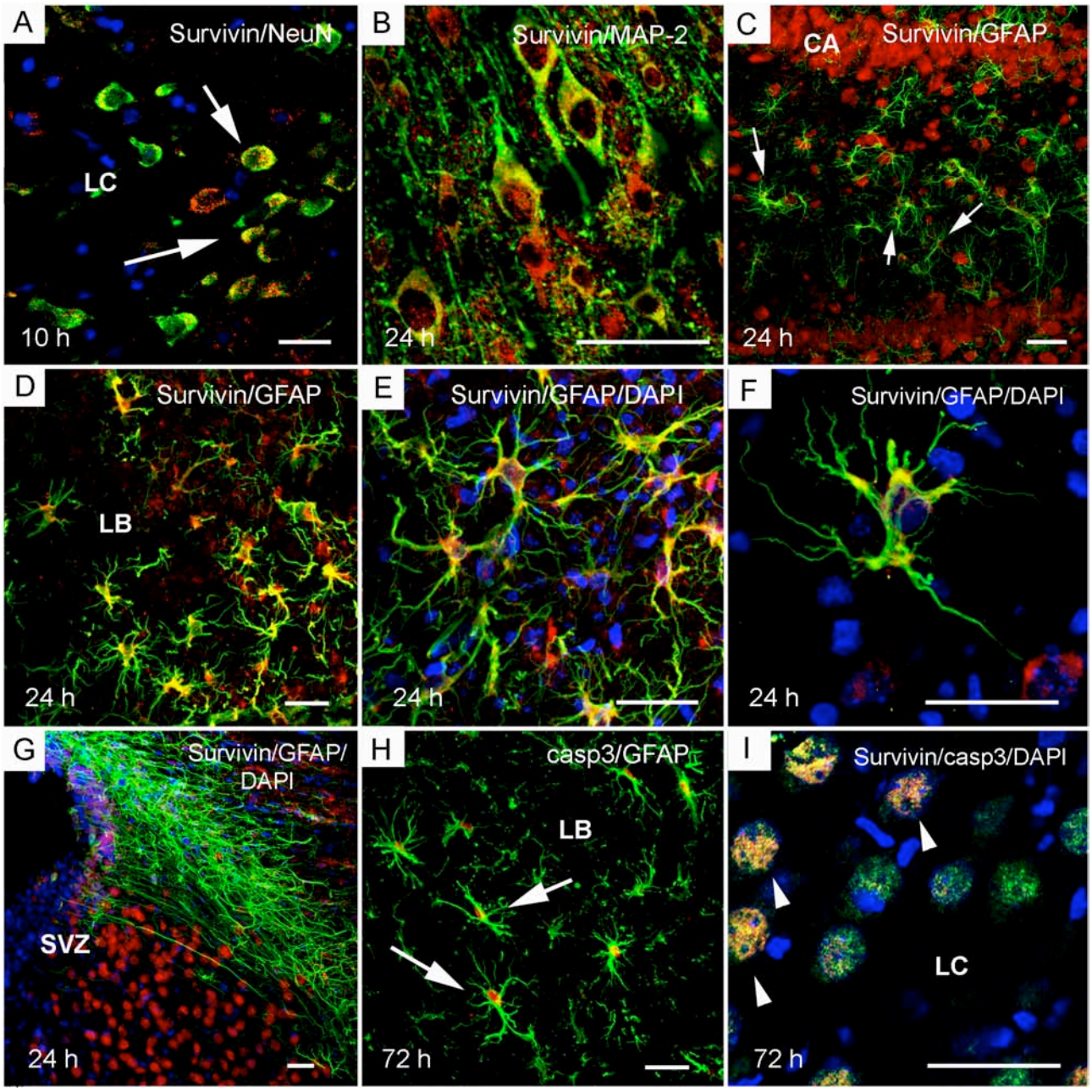


Figure 2. *Confocal immunofluorescence showing survivin (red) in neurons (NeuN and MAP-2; green in A and B, respectively) and astrocytes (GFAP; C-G) or astrocytes colocalizing with cleaved caspase-3 (casp3; green, H) or survivin with cleaved caspase-3 (casp3; green I) and DAPI nuclear staining is shown in blue.* Survivin positive neurons are seen in damaged cortical areas at 10 hours as shown by NeuN double labeling in the lesion border (A), or in the lesion core with MAP-2 (B) at 24 hpl. Other survivin expressing areas include the hippocampal pyramidal layer where survivin positive cells colocalize with GFAP (arrows in C). However, the main area showing survivin expression is the lesion border, where survivin staining is cytoplasmatic and localized to GFAP positive (D) and in the degenerating damaged cortex, both in the lesion core where survivin is also found in reactive astrocytes at 24 hpl (E and F). High survivin expression is also found in the subventricular zone, a germinative area where a few cells colocalized with GFAP (G). Cleaved caspase-3 in astrocytes (arrows in H) and with survivin in cortical area at 72 hpl, which colocalized in the nucleus (arrowheads) of cells in the cortical lesion core (I). Abbreviations: CA (cornu ammonis); cc (corpus callosum); cx (cortical area); LB (lesion border); LC (lesion core); SVZ (subventricular zone). Scale bars = 20 µm.

Survivin is expressed in neurons and astrocytes in regions showing cleaved caspase-3

Survivin was observed in the soma of MAP-2+ and NeuN+ neurons in the cortical lesion border (Fig. 2A) and in the lesion core (Fig. 2B), mainly at 10-24 hours post-lesion. However, at longer survival times, neuronal survivin expression also expanded to adjacent non-damaged cortical areas (data not shown). Neuronal survivin expression was also seen in the CA field of the hippocampus (Fig. 2C) from 10 to 72 hours post-lesion, when GFAP+ astroglial cells expressing nuclear survivin were also seen (Fig. 2C), mainly at 72 hours post-lesion, when 48.7±7.4% of survivin+ cells were astrocytes in the hippocampus (Fig. 3). In the damaged cortex, astroglial survivin was found in the nucleus and/or cytoplasm (Fig. 2D-F) of reactive astrocytes present in the lesion core (Fig. 2E) and the lesion border (Fig. 2F), mainly at 72 hours post-lesion, when astrocytes were 35.6±5.2% and 37.9±3.7% of all survivin+ cells, respectively (Fig. 3). Noteworthy, the highest percentage of survivin+/GFAP+ was found in the corpus callosum, where astrocytes represented 45.8±2.4% of all survivin+ cells at 24 hours post-lesion, and 61.9±7,8% at 72

hours post-lesion (Fig. 3). Finally, there was a considerable amount of survivin+ cells in the subventricular zone (SVZ), one of the neurogenic niches, where a few cells also showed colocalzation with GFAP (Fig. 2F).
The analysis of survivin and cleaved caspase-3 showed that at 24 and 72 hours post-lesion, when maximum astroglial caspase-3 occurs (Fig. 2H) (Acarin et al., 2007), colocalization was mainly observed in cells showing survivin expression in the nucleus (Fig. 2I), in the cortical lesion core at both times and in the lesion border at 72 hours post-lesion (Table 1). Double-labeled survivin+/caspase-3+ cells were also found in the hippocampus and the corpus callosum.

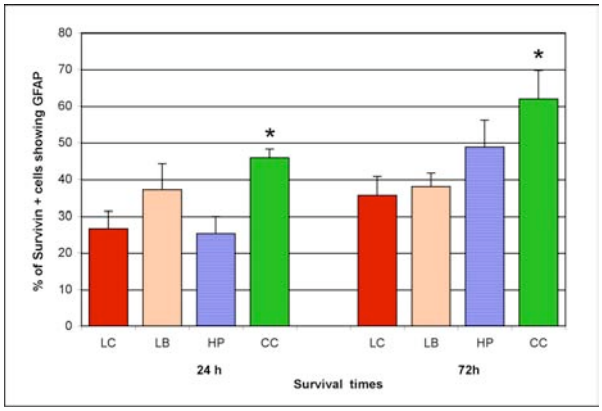


Figure 3. *Percentage of survivin positive cells showing GFAP colocalization in the cortical lesion core (LC), lesion border (LB), hippocampus (HP) and corpus callosum (CC) at 24 and 72 hours post-lesion. Data is shown as mean ± sem. Although maximun colocalization is found in the cc at both times (*, p<0.05) all areas show survivin + astrocytes, mainly at 72 h post-lesion.*

cIAP-2 is expressed in neurons mainly in the lesioned cortex

In the CL hemisphere, some cIAP-2 positive cells were observed (data not shown) but expression was limited. In the IL hemisphere, cells expressing cIAP-2 were observed in the damaged cortex, the adjacent corpus callosum, and the hippocampus to a lesser extent. In the damaged cortex and adjacent white matter, cIAP-2 expression was first observed at 4 hours post-lesion, but increased at 10-24 hours post-lesion, when cytoplasmic cIAP-2 labeling was found in the deeper cortical layers (layer VI) at the level of the lesion core and in the cingulum of the corpus callosum (Fig. 4A). Noteworthy, cIAP-2 labeling was generally more intense in the cortical lesion border and layer VI than in the lesion core itself (Fig. 4A, B). It was mainly found in cells showing neuronal morphology, which did not correlate with GFAP labeling (Fig. 4C). However, at 7 days post-lesion, when the glial scar is formed, a few reactive astrocytes showed cIAP-2 expression in the corpus callosum (Fig. 4D) and in the cortex (Fig. 4E). Finally, colocalization between cIAP-2 and cleaved caspase-3 (Fig. 4F) was observed in some cells within the lesion core and in the lesion border at early survival times (Table 1), mainly in cells showing neuronal morphology.

HSP25/27 is expressed in reactive astrocytes in the damaged cortex and strongly colocalizes with cleaved caspase-3

In the CL hemisphere of lesioned animals and in both hemispheres of saline-injected animals, expression of HSP25/27 was very low (data not shown). However, in the IL hemisphere of lesioned animals, HSP25/27 was mainly present in the damaged cortex and adjacent white matter, although some HSP25/27+ cells were also observed in the striatum and the hippocampus (Fig. 5). In the cortex, HSP25/27 was first induced at 10 hours post-lesion, when labeling was seen in the lesion core, the lesion border and in the adjacent non-damaged cortex. At 24-72 hours post-lesion, HSP25/27 was strongly increased (Fig. 5A) and was mainly expressed in astroglial cells (Fig 5A—F, 6A, B, 7), although some neuronal cells in the lesion border (Fig. 5A) and in the hippocampus were also HSP25/27+. In the cortex, HSP25/27+ astrocytes were observed in the lesion core (Fig. 6A-B, 7), where they represented 68.4±4.7% of all HSP25/27+ cells; in the lesion border, mainly at 24 hours post-lesion in the medial areas (Fig. 5A, B), and in cortical layer VI (Fig. 5A, D). Moreover, 52.6±8.2% of all HSP25/27+ cells in the corpus callosum were reactive astrocytes (Fig. 5A, C, 6C, 7) while in the hippocampus astrocytes constituted only the 17.8±3.1% of cells (Fig. 5A, E, 7). HSP25/27 labeling in reactive astrocytes was principally cytoplasmatic and displayed thick and fine astroglial branches (Fig. 5F). Noteworthy, not all GFAP+ reactive astrocytes and vimentin+ astrocytes expressed HSP25/27 (Fig. 6 A, B). From 7 days post-lesion, astroglial HSP25/27 expression remained in the glial scar, corpus callosum (Fig. 6C) and in cortical layer VI. The colocalization analysis between HSP25/27 and cleaved caspase-3 showed a strong correlation of both markers (Table 1), with a very high proportion of double labeled cells in the damaged cortex (Fig. 6D, E) and in the adjacent corpus callosum, mainly at 24-72 hours post-lesion, when caspase-3 is mostly located in reactive astrocytes (Acarin et al., 2007).

Table 1. *Summary of colocalization between survivin, HSP25/27, and cIAP-2 with cleaved caspase-3 at different survival times after excitotoxic damage.*

	Cleaved Caspase-3					
	Survivin		cIAP-2		HSP25/27	
	24 h	72 h	24 h	72 h	24 h	72 h
Cortical lesion core	++++	++	++	+	++++	+++
Cortical lesion border	+	+++	+	++	++	+++
Hippocampus	++	+	-/+	-	-/+	+
Corpus callosum	+	++	-/+	+	+++	++++

The table shows semi quantitative analysis of the colocalization of cleaved caspase-3 with survivin, HSP25/27, and cIAP-2 in subfields of the damaged cortex, hippocampus and corpus callosum, determined by a qualitative analysis of double immunoreactive cells at 24 and 72 hours after NMDA injection. -, no double labeled cells; -/+, very few double cells; +, scattered labeled cells; ++, approximately half of cells double labeled; +++, the majority of labeled cells; ++++, virtually all cells double labeled.

HSC70/HSP70 is induced in cortical neurons and shows poor correlation with caspase-3 activation

HSC70/HSP70 was transiently induced in the IL cortex and mostly found in layers I-IV (Fig. 6G), although scattered HSC70/HSP70+ cells were observed in the hippocampus. In the cortex, HSC70/HSP70 immunoreactivity increased at 24-72 hours post-lesion, mainly in pyramidal cells (Fig. 6G) and sometimes extended to the lesion border. Colocalization studies showed no correlation between HSC70/HSP70 and GFAP immunostaining (Fig. 6G), and only an occasional occurrence of HSC70/HSP70+/caspase-3+ double labeled cells (Fig. 6H).

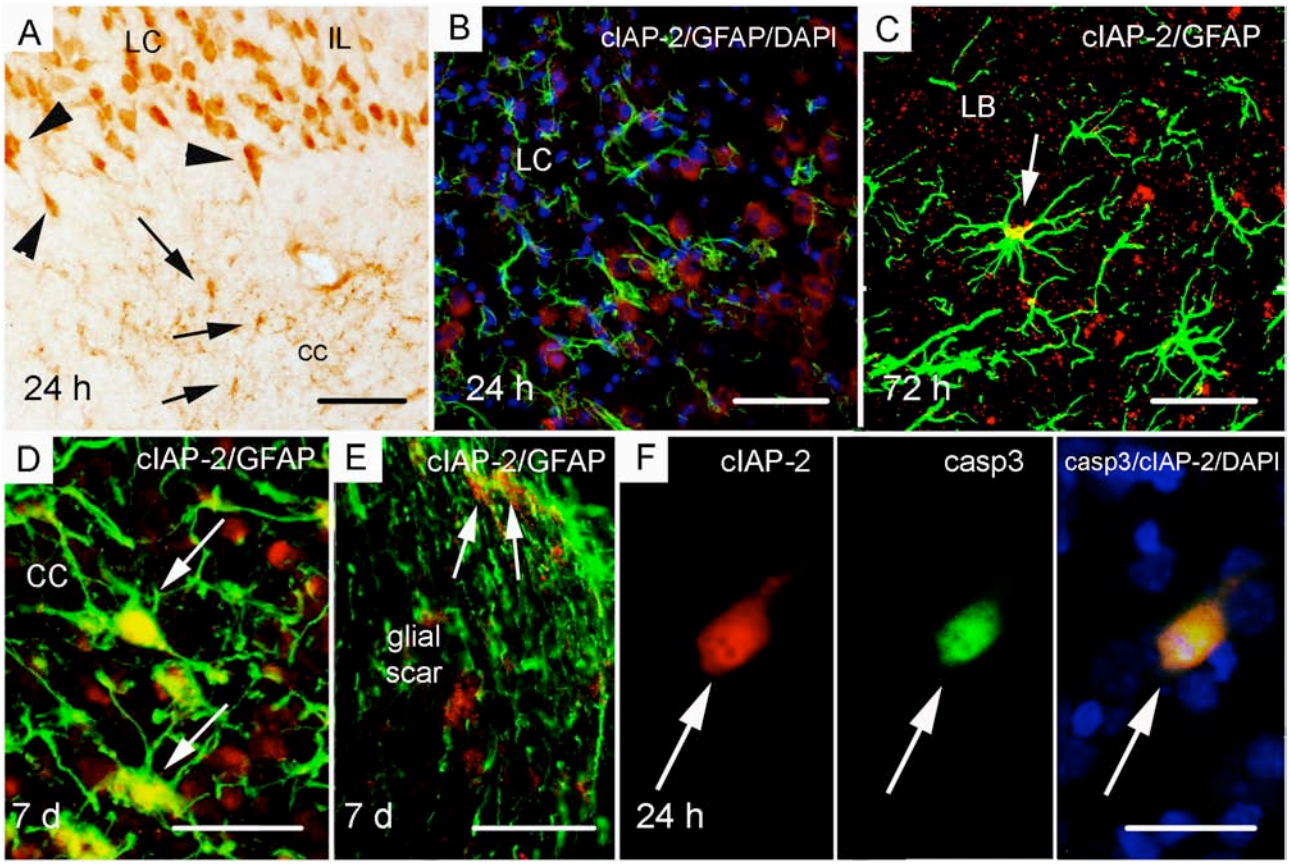


Figure 4. *Light microscopy showing cortical cIAP-2 (A), and confocal immunofluorescence cIAP-2 (red; B-F) containing astrocytes (GFAP; green; B-E) and cleaved caspase-3 (casp3; green; F).* In the ipsilateral cortex, cIAP-2 is expressed in neuronal soma (arrowheads in A) or in cells showing a branched morphology (arrows in A) in the corpus callosum. In the cortical lesion core, cIAP-2 positive cells do not colocalize with GFAP at 24 hours post-lesion (B), and only a small amount of astrocytes shown cIAP-2 in the cortical lesion border at 72 hours post-lesion (arrow in C). At 7 days post-lesion, cIAP-2 is observed in astrocytes in the corpus callosum (arrow in D), and the glial scar (arrow in E). In the cortical lesion core, cIAP-2 was expressed in the nucleus and cytosol of cells showing nuclear cleaved caspase-3 (arrow in F). DAPI staining for nuclei. Abbreviations: cc (corpus callosum); cx (cortical area); LB (lesion border); LC (lesion core). Scale bars = 20 μm.

DISCUSSION

In this study we describe that different endogenous anti-apoptotic proteins, mainly survivin, of the IAP family of proteins, and Hsp25 of the HSP family of chaperones, colocalize with active caspase-3 in reactive astrocytes following excitotoxic brain damage. In addition, the anti-apoptotic protein cIAP-2 was also expressed in corpus callosum and glial scar reactive astrocytes at 7 dpl when astroglial response peaks in this lesion model (Acarin et al., 1999b). As colocalization of these protective molecules is found at different survival times when caspase-3 activity and astroglial cell death does not occur (Acarin et al., 2007), our findings suggest that these proteins may be partly responsible for the blockade of caspase-3 activity downstream of its cleavage, as has been previously described in other cell types (Vassina et al., 2006), and might contribute, in this way, to the protective mechanism developed by reactive astroglial cells in order to cope with tissue damage and oxidative stress after brain injury.

Survivin and cIAP-2 protect surviving astrocytes showing caspase-3 cleavage

Several members of the IAP family of proteins have been shown to inhibit cleavage-activated caspase-3 directly, blocking apoptotic effector events downstream of this caspase (Peng et al., 2005) and providing safeguard mechanisms against minimum activation of the caspase apoptosis cascade by setting up an endogenous threshold level for caspase activation.

Survivin has different splicing variants which have been shown to be able to inhibit caspase function by two different mechanisms: through direct blocking of the enzymatic activity of caspase-3 once it has been proteolytically activated (Tamm et al., 1998; Shin et al., 2001), and through the ubiquitination of caspase-3, leading to its degradation by the proteasome (Vegran et al., 2005), therefore being a suitable candidate for the anti-apoptotic protective mechanisms developed by glial cells after brain injury. Survivin expression has long been studied in astrocytomas and other glia-derived tumors (Kajiwara et al., 2003), in which it has been shown to play an important role in tumor progression and malignancy. However, very few studies have analysed survivin expression after acute brain damage (Johnson and Howerth, 2004; Johnson et al., 2005). To our knowledge, this is the first work reporting survivin expression after immature brain injury, although expression of this cell death regulator plays an important role in the anti-apoptotic cell protection mechanism of neuronal precursor cells during CNS embryogenesis when its deletion induces increased caspase-3 and caspase-9 activities massive neuronal death due to deregulated apoptosis, and death early in life (Jiang et al., 2005). We here suggest that survivin may also play a role in neuronal apoptosis regulation following acute excitotoxicity in the immature brain, as it was found in neuronal cells of the injured cortex and adjacent hippocampus where we have described neuronal activation of the mitochondrial pathway through caspase-9 and TUNEL labeling (Acarin et al., 2007; Villapol et al., 2007).

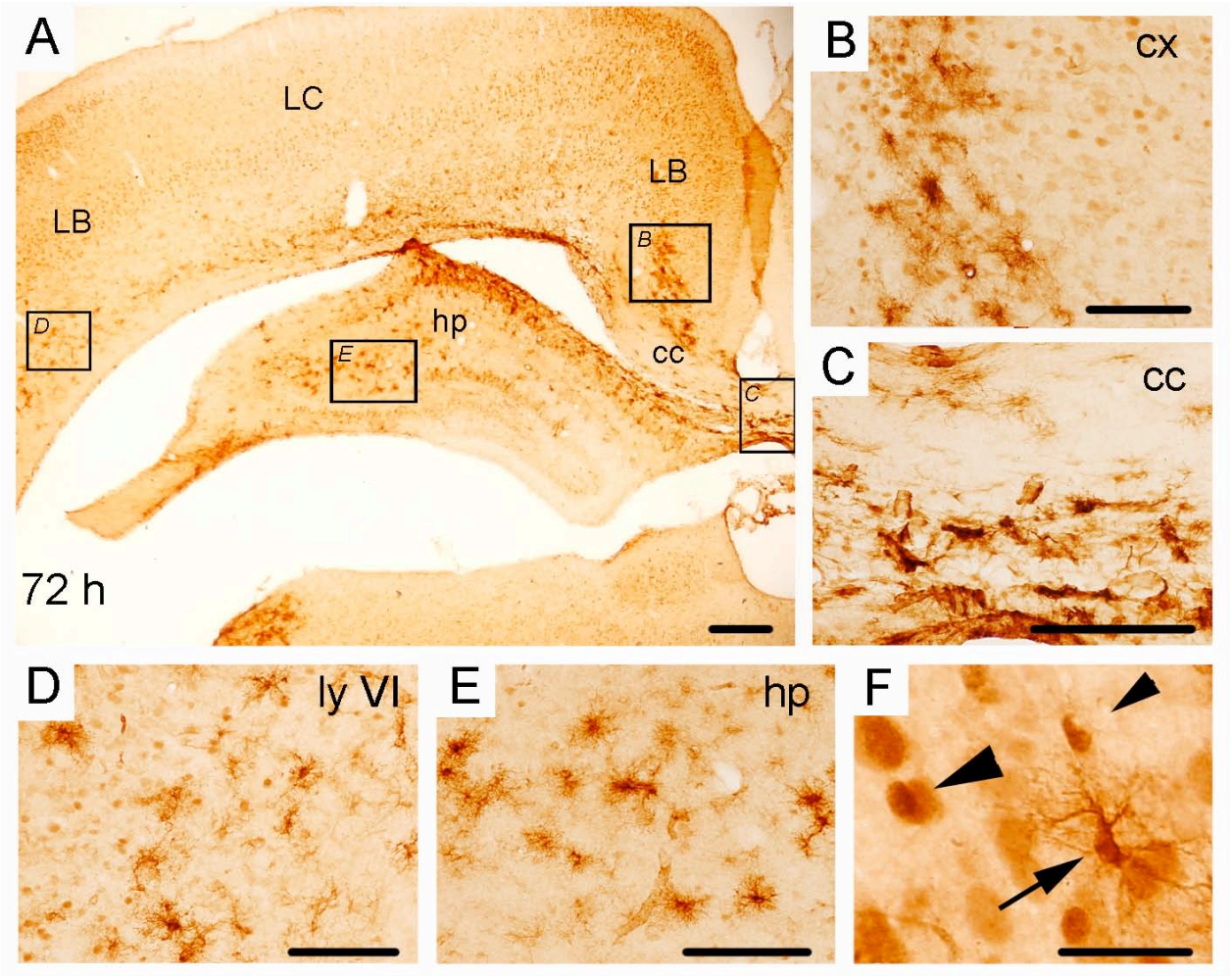


Figure 5. HSP25/27 distribution in the injured hemisphere at 72 hours post-lesion (A). HSP25/27 + cells predominate in the medial cortex (B), medial corpus callosum (C), cortical layer VI (D), and hippocampal CA (E). HSP25/27 positive cells show ramified morphology (arrows in F) or nuclear morphology (arrowheads in F) in the cortical neurodegenerative area. Abbreviations: CA (cornu ammonis); cc (corpus callosum); cx (cortical area); hp (hippocampus); LB (lesion border); LC (lesion core); ly VI (cortical layer VI). Scale bars = 20 µm.

In addition, we have observed a significant colocalization of this IAP in reactive astrocytes showing cleaved caspase-3, in agreement with the pattern of survivin expression following adult traumatic brain injury (Johnson and Howerth, 2004; Johnson et al., 2005), where astroglial survivin expression was correlated with the lower incidence of TUNEL labeling in this glial cell population. Interestingly, survivin gene expression has been recently shown to be regulated by the Signal Transducer and Activator of Transcription 3 (STAT3) (Gritsko et al., 2006) a transcription factor activated in astrocytes from 2 hours until 14 dpl after postnatal excitotoxicity (Acarin et al., 1998, 2000), which could account for astroglial survivin expression.

Besides its role in cell death regulation, survivin is largely known for its function as a cell division regulator in cancer cells (Altieri, 2004, 2006), showing cell cycle expression regulation with a robust expression in G2/M phase (Li et al., 1998). Following postnatal excitotoxicity, we here describe that survivin is found in the germinal SVZ where proliferating precursor cells are found (Faiz et al., 2005). However, it should be noted that cleaved caspase-3 is also found in these progenitor cells (S. Villapol, unpublished results), and survivin could also play an anti-apoptotic role in these cells. Finally, it is interesting to note that survivin is here described to colocalize with cleaved caspase-3 in reactive astrocytes, but this caspase does not correlate with 5'-bromodeoxyuridine (BrdU) incorporation in astrocytes in this model (Acarin et al., 2007), in agreement with the results by Johnson et al. following adult traumatic injury, reporting only 12% colocalization between survivin+ astrocytes and the proliferating cell nuclear antigen (PCNA), although the majority of proliferating cells are astrocytes (Johnson et al., 2004). In general, these findings suggest an outstanding anti-apoptotic role of survivin, more than the control of proliferation in reactive astrocytes.

cIAP-2 is another member of the IAP family which is also considered a physiologically relevant inhibitor of caspase-3 (Nishihara et al., 2003) by means of its high affinity towards the cleaved form of caspase-3, but not its non-processed precursor (Roy et al., 1997). We here describe cIAP-2 expression mainly in neuronal cells following immature excitotoxicity, as has been previously reported after trauma in the adult (Keane et al., 2001), however colocalization between cleaved caspase-3 and cIAP-2 was not as relevant as the other anti-apoptotic proteins evaluated. Interestingly, cIAP-2 was specifically expressed in the cytosol of glial scar reactive astrocytes at 7 days post-lesion, but not throughout the evolution of the astroglial response. To our knowledge, reports on the astroglial expression of cIAP-2 are very scarce; cIAP-2 is expressed in astrocytomas (Liu et al., 2006), but only recently it has been reported in reactive astrocytes following treatment with the anti-apoptotic factor granulocyte colony stimulating factor (G-CSF) after ischemia (Solaroglu et al., 2006).

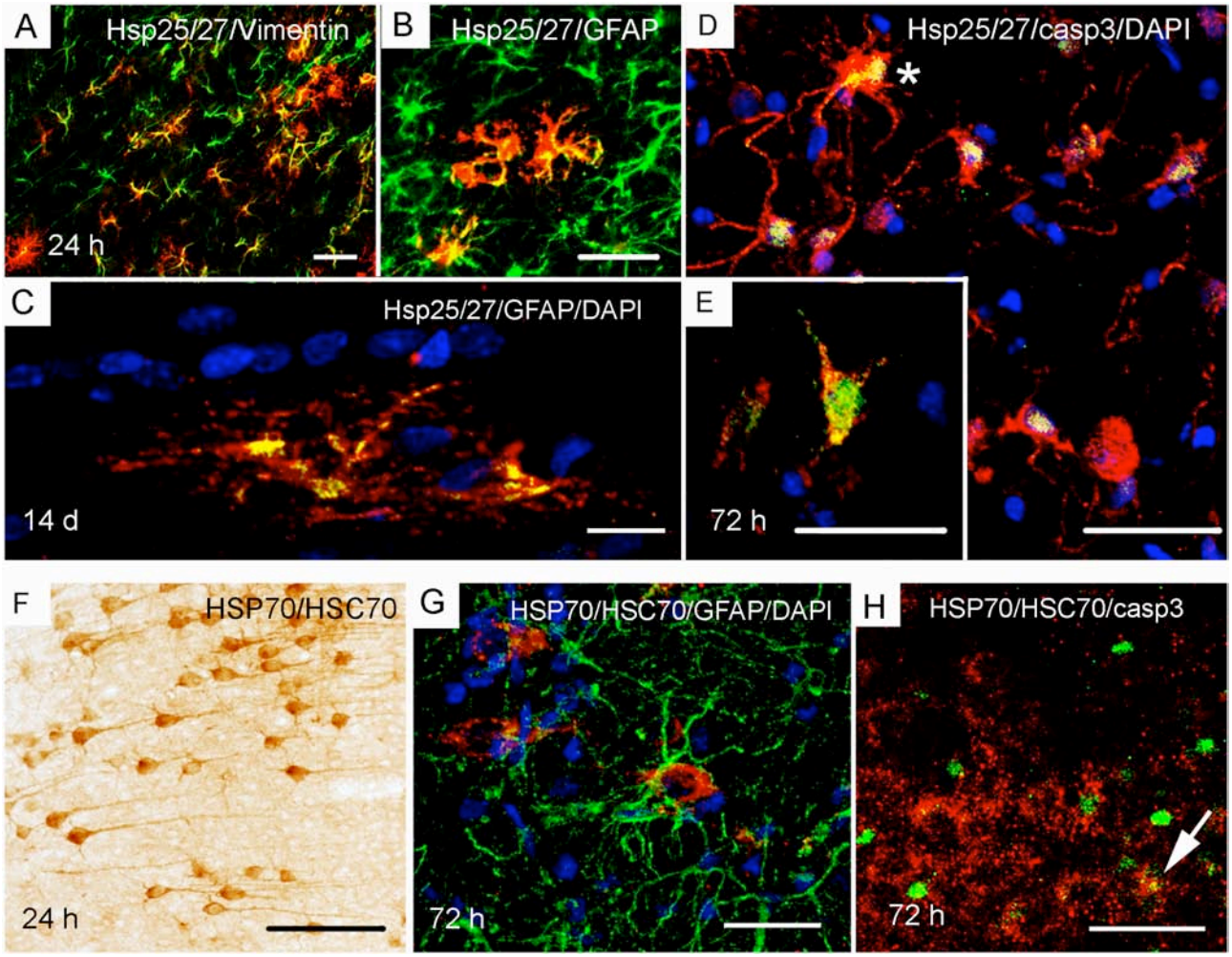


Figure 6. *Confocal immunofluorescence showing cortical HSP25/27 (red), Vimentin (green in A), GFAP (green in B and C), and cleaved caspase-3 (casp3; green; D and E).* In the injured cortex, at 24 hours post-lesion, HSP25/27-labeled cells are identified as reactive astrocytes by their labeling with vimentin (A) and GFAP (B) at 24 hpl. Astroglial cells become further hypertrophied at longer survival times, 14 days, and they also express HSP25/27 in the medial corpus callosum (C). HSP25/27 positive astrocytes are also seen in the lesion border at 72 hours post-lesion, where numerous cells shown nuclear cleaved caspase-3 and HSP25/27 in the cytoplasm (D) and in the nucleus (E). Hsc70/Hsp70 is found in pyramidal cortical neurons (F) and does not colocalize with GFAP (G), and only very occasionally colocalize with cleaved caspase-3 (arrows in H). Scale bars = 20 μm.

HSP25/27 may provide anti-apoptotic and cytoskeletal protection to reactive astrocytes

A few years ago we described that a subpopulation of reactive astrocytes induced HSP25/27 expression after acute damage to the immature rat brain, both in traumatic injury (Sanz et al., 2001) and excitotoxicity (Acarin et al., 2002). We here show that this molecular chaperone strongly colocalizes with the presence of cleaved caspase-3 in the absence of cell death, therefore suggesting an anti-apoptotic role of HSP25/27 in astrocytes, as has been demonstrated in other cell types (Concannon et al., 2003). It should be noted that of all molecules analyzed in this study, HSP25/27 is the anti-apoptotic protein that shows the highest expression in astrocytes and the strongest colocalization with cleaved caspase-3. It is now established that several types of cells induced to accumulate HSPs become more tolerant to a variety of cytotoxic insults, supporting the idea that HSPs are able to prevent cell death and promote survival (Latchman, 2004; Sreedhar and Csermely, 2004). In particular, several mechanisms have been proposed to account for the cytoprotective effects of HSP25/27, including its role as molecular chaperone facilitating repair/destruction of damaged proteins, modulation of oxidative stress, direct interaction with key components of the caspase apoptotic cascade, and protection of cytoskeletal components (Concannon et al., 2003). As a chaperone with a well-known ability to interact with different proteins, HSP25/27 has been shown to inhibit the intrinsic mitochondrial apoptotic pathway through interaction with cytochrome c after its release from the mitochondria, and pro-caspase-3, preventing the correct formation of the apoptosome complex (Pandey et al., 2000; Concannon et al., 2001; Garrido et al., 2001; Paul et al., 2002), and inhibit the death receptor extrinsic caspase pathway (Mehlen et al., 1995; Mehlen et al., 1996; Arrigo et al., 2007). Noteworthy, it has recently been shown that HSP27 is able to directly associate with caspase-3, inhibiting caspase-3 activity and preventing apoptosis (Voss et al., 2007), which could explain the colocalization between HSP25/27 and caspase-3 we here describe in reactive astrocytes. In addition to its anti-apoptotic properties, HSP25/27 has long been recognized as a potent regulator of cytoskeletal dynamics potentiating stabilization of actin microfilaments by preventing their disaggregation following stressful conditions (Guay et al., 1997; Concannon et al., 2003).

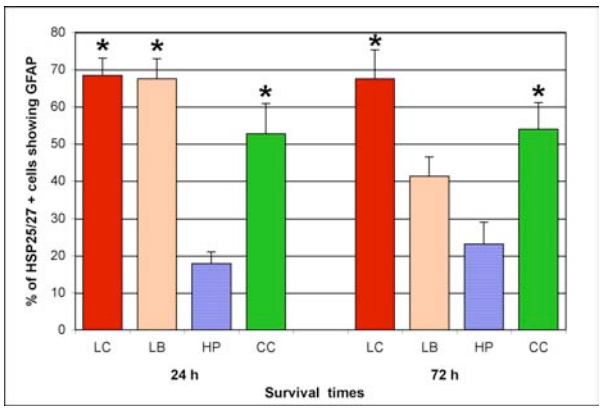


Figure 7. Percentage of HSP25/27 + cells showing GFAP colocalization in the cortical lesion core (LC), lesion border (LB), hippocampus (HP) and corpus callosum (CC) at 24 and 72 hours post-lesion. Data is shown as mean ± sem. Maximum amount of double labeled cells is found in the LC, LB and CC at 24 hpl (*, $p<0.05$) and percentage of colocalization is maintained at the LC and CC, but slightly diminished in the LB. Colocalization is low in the hippocampus at both times.

Furthermore, HSP25/27 can also interact with other cytoskeletal components, including several intermediate filaments like GFAP and vimentin in astroglial cells (Perng et al., 1999; Lee et al., 2005). In cell lines, HSP27 can interact with both the intermediate filament and the soluble intermediate filament proteins within the cell cytoplasm, preventing filaments from forming non-covalent filament-filament interactions that could induce pathological aggregations (Perng et al., 1999), and can physically associate with vimentin, avoiding morphological alterations of vimentin-containing filaments and disruption of intermediate filament networks (Lee et al., 2005). Interestingly, and in relation to this protective role, we have previously described, after postnatal excitotoxic damage, caspase targeting of GFAP by demonstrating the presence of caspase-cleaved GFAP fragments within the first 24 hours post-lesion (Acarin et al., 2007), when astroglial expression of HSP25/27 protein is still very low (Acarin et al., 2002). In this regard, we here suggest that following immature brain damage, HSP25/27 plays a key role in astroglial cell survival by blocking apoptotic cascades and maintaining the integrity of astroglial cytoskeleton, promoting cell stabilization.

Finally, the low correlation of HSC/HSP70 and caspase-3 in reactive astrocytes also deserves discussion. The neuroprotective role of HSP70 after acute damage has been extensively documented in the last decade including in the immature brain, where its overexpression induces decreased caspase-8 and caspase-9 activation, therefore targeting both the intrinsic and extrinsic caspase apoptotic pathway and other caspase-independent mechanisms (Matsumori et al., 2005; Matsumori et al., 2006). In addition, constitutive HSC70 modulates caspase-3 activation in the developing brain (Rubio et al., 2002), and HSP70 interacts directly with cleaved caspase-3, preventing its activity (Jaattela et al., 1998). Besides its expression in neurons, several studies have reported HSP70 expression in reactive astrocytes following ischemia, both in the adult and the early postnatal brain (Sharp et al., 1991; Sharp et al., 1993; Bergeron et al., 1996; Renolleau et al., 1997; Giffard et al., 2004), and astroglial HSP70 has been implicated in the cellular protection from mitochondrial damage induced by oxygen glucose deprivation, heat shock and acidosis (Narasimhan et al., 1996; Papadopoulos et al., 1996; Xu and Giffard, 1997; Ouyang et al., 2006).

In conclusion, we here present evidence that mainly survivin and HSP25/27, and cIAP-2 to a lower extent, are strong candidates responsible for the lack of caspase-3 activity and cell death in reactive astrocytes showing cleaved caspase-3, providing protective mechanisms to astroglial cells after immature brain damage.

REFERENCES

Acarin L, Gonzalez B, Castellano B (1998) Stat3 and NFkappaB glial expression after excitotoxic damage to the postnatal brain. Neuroreport 9:2869-2873.

Acarin L, Gonzalez B, Castellano B (2000) STAT3 and NFkappaB activation precedes glial reactivity in the excitotoxically injured young cortex but not in the corresponding distal thalamic nuclei. J Neuropathol Exp Neurol 59:151-163.

Acarin L, Gonzalez B, Castellano B (2001) Glial activation in the immature rat brain: implication of inflammatory transcription factors and cytokine expression. Prog Brain Res 132:375-389.

Acarin L, Gonzalez B, Castro AJ, Castellano B (1999a) Primary cortical glial reaction versus secondary thalamic glial response in the excitotoxically injured young brain: microglial/macrophage response and major histocompatibility complex class I and II expression. Neuroscience 89:549-565.

Acarin L, Paris J, Gonzalez B, Castellano B (2002) Glial expression of small heat shock proteins following an excitotoxic lesion in the immature rat brain. Glia 38:1-14.

Acarin L, Gonzalez B, Hidalgo J, Castro AJ, Castellano B (1999b) Primary cortical glial reaction versus secondary thalamic glial response in the excitotoxically injured young brain: astroglial response and metallothionein expression. Neuroscience 92:827-839.

Acarin L, Villapol S, Faiz M, Rohn TT, Castellano B, Gonzalez B (2007) Caspase-3 activation in astrocytes following postnatal excitotoxic damage correlates with cytoskeletal remodeling but not with cell death or proliferation. Glia.

Altieri DC (2004) Molecular circuits of apoptosis regulation and cell division control: the survivin paradigm. J Cell Biochem 92:656-663.

Altieri DC (2006) The case for survivin as a regulator of microtubule dynamics and cell-death decisions. Curr Opin Cell Biol 18:609-615.

Anguelova E, Smirnova T (2000) Differential expression of small heat shock protein 27 in the rat hippocampus and septum after fimbria-fornix lesion. Neurosci Lett 280:99-102.

Arrigo AP, Simon S, Gibert B, Kretz-Remy C, Nivon M, Czekalla A, Guillet D, Moulin M, Diaz-Latoud C, Vicart P (2007) Hsp27 (HspB1) and alphaB-crystallin (HspB5) as therapeutic targets. FEBS Lett.

Bergeron M, Mivechi NF, Giaccia AJ, Giffard RG (1996) Mechanism of heat shock protein 72 induction in primary cultured astrocytes after oxygen-glucose deprivation. Neurol Res 18:64-72.

Clem RJ, Hardwick JM, Miller LK (1996) Anti-apoptotic genes of baculoviruses. Cell Death Differ 3:9-16.

Concannon CG, Orrenius S, Samali A (2001) Hsp27 inhibits cytochrome c-mediated caspase activation by sequestering both pro-caspase-3 and cytochrome c. Gene Expr 9:195-201.

Concannon CG, Gorman AM, Samali A (2003) On the role of Hsp27 in regulating apoptosis. Apoptosis 8:61-70.

Currie RW, Ellison JA, White RF, Feuerstein GZ, Wang XK, Barone FC (2000) Benign focal ischemic preconditioning induces neuronal Hsp70 and prolonged astrogliosis with expression of Hsp27. Brain Res 863:169-181.

Faiz M, Acarin L, Castellano B, Gonzalez B (2005) Proliferation dynamics of germinative zone cells in the intact and excitotoxically lesioned postnatal rat brain. BMC Neurosci 6:26.

Ferrer I, Planas AM (2003) Signaling of cell death and cell survival following focal cerebral ischemia: life and death struggle in the penumbra. J Neuropathol Exp Neurol 62:329-339.

Garrido C, Gurbuxani S, Ravagnan L, Kroemer G (2001) Heat shock proteins: endogenous modulators of apoptotic cell death. Biochem Biophys Res Commun 286:433-442.

Giffard RG, Xu L, Zhao H, Carrico W, Ouyang Y, Qiao Y, Sapolsky R, Steinberg G, Hu B, Yenari MA (2004) Chaperones, protein aggregation, and brain protection from hypoxic/ischemic injury. J Exp Biol 207:3213-3220.

Gritsko T, Williams A, Turkson J, Kaneko S, Bowman T, Huang M, Nam S, Eweis I, Diaz N, Sullivan D, Yoder S, Enkemann S, Eschrich S, Lee JH, Beam CA, Cheng J, Minton S, Muro-Cacho CA, Jove R (2006) Persistent activation of

stat3 signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells. Clin Cancer Res 12:11-19.

Guay J, Lambert H, Gingras-Breton G, Lavoie JN, Huot J, Landry J (1997) Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. J Cell Sci 110 (Pt 3):357-368.

Jaattela M, Wissing D, Kokholm K, Kallunki T, Egeblad M (1998) Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. Embo J 17:6124-6134.

Jiang Y, de Bruin A, Caldas H, Fangusaro J, Hayes J, Conway EM, Robinson ML, Altura RA (2005) Essential role for survivin in early brain development. J Neurosci 25:6962-6970.

Johnson EA, Svetlov SI, Wang KK, Hayes RL, Pineda JA (2005) Cell-specific DNA fragmentation may be attenuated by a survivin-dependent mechanism after traumatic brain injury in rats. Exp Brain Res 167:17-26.

Johnson EA, Svetlov SI, Pike BR, Tolentino PJ, Shaw G, Wang KK, Hayes RL, Pineda JA (2004) Cell-specific upregulation of survivin after experimental traumatic brain injury in rats. J Neurotrauma 21:1183-1195.

Johnson ME, Howerth EW (2004) Survivin: a bifunctional inhibitor of apoptosis protein. Vet Pathol 41:599-607.

Kajiwara Y, Yamasaki F, Hama S, Yahara K, Yoshioka H, Sugiyama K, Arita K, Kurisu K (2003) Expression of survivin in astrocytic tumors: correlation with malignant grade and prognosis. Cancer 97:1077-1083.

Kato H, Kogure K, Liu XH, Araki T, Kato K, Itoyama Y (1995) Immunohistochemical localization of the low molecular weight stress protein HSP27 following focal cerebral ischemia in the rat. Brain Res 679:1-7.

Keane RW, Kraydieh S, Lotocki G, Alonso OF, Aldana P, Dietrich WD (2001) Apoptotic and antiapoptotic mechanisms after traumatic brain injury. J Cereb Blood Flow Metab 21:1189-1198.

Latchman DS (2004) Protective effect of heat shock proteins in the nervous system. Curr Neurovasc Res 1:21-27.

Lee JS, Zhang MH, Yun EK, Geum D, Kim K, Kim TH, Lim YS, Seo JS (2005) Heat shock protein 27 interacts with vimentin and prevents insolubilization of vimentin subunits induced by cadmium. Exp Mol Med 37:427-435.

Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, Altieri DC (1998) Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 396:580-584.

Liu X, Chen N, Wang X, He Y, Chen X, Huang Y, Yin W, Zhou Q (2006) Apoptosis and proliferation markers in diffusely infiltrating astrocytomas: profiling of 17 molecules. J Neuropathol Exp Neurol 65:905-913.

Matsumori Y, Northington FJ, Hong SM, Kayama T, Sheldon RA, Vexler ZS, Ferriero DM, Weinstein PR, Liu J (2006) Reduction of caspase-8 and -9 cleavage is associated with increased c-FLIP and increased binding of Apaf-1 and Hsp70 after neonatal hypoxic/ischemic injury in mice overexpressing Hsp70. Stroke 37:507-512.

Matsumori Y, Hong SM, Aoyama K, Fan Y, Kayama T, Sheldon RA, Vexler ZS, Ferriero DM, Weinstein PR, Liu J (2005) Hsp70 overexpression sequesters AIF and reduces neonatal hypoxic/ischemic brain injury. J Cereb Blood Flow Metab 25:899-910.

McLaughlin B (2004) The kinder side of killer proteases: caspase activation contributes to neuroprotection and CNS remodeling. Apoptosis 9:111-121.

Mehlen P, Schulze-Osthoff K, Arrigo AP (1996) Small stress proteins as novel regulators of apoptosis. Heat shock protein 27 blocks Fas/APO-1- and staurosporine-induced cell death. J Biol Chem 271:16510-16514.

Mehlen P, Kretz-Remy C, Briolay J, Fostan P, Mirault ME, Arrigo AP (1995) Intracellular reactive oxygen species as apparent modulators of heat-shock protein 27 (hsp27) structural organization and phosphorylation in basal and tumour necrosis factor alpha-treated T47D human carcinoma cells. Biochem J 312 (Pt 2):367-375.

Narasimhan P, Swanson RA, Sagar SM, Sharp FR (1996) Astrocyte survival and HSP70 heat shock protein induction following heat shock and acidosis. Glia 17:147-159.

Nishihara H, Kizaka-Kondoh S, Insel PA, Eckmann L (2003) Inhibition of apoptosis in normal and transformed intestinal epithelial cells by cAMP through induction of inhibitor of apoptosis protein (IAP)-2. Proc Natl Acad Sci U S A 100:8921-8926.

Nishino K, Nowak TS, Jr. (2004) Time course and cellular distribution of hsp27 and hsp72 stress protein expression in a quantitative gerbil model of ischemic injury and tolerance: thresholds for hsp72 induction and hilar lesioning in the context of ischemic preconditioning. J Cereb Blood Flow Metab 24:167-178.

Ouyang YB, Xu LJ, Sun YJ, Giffard RG (2006) Overexpression of inducible heat shock protein 70 and its mutants in astrocytes is associated with maintenance of mitochondrial physiology during glucose deprivation stress. Cell Stress Chaperones 11:180-186.

Pandey P, Farber R, Nakazawa A, Kumar S, Bharti A, Nalin C, Weichselbaum R, Kufe D, Kharbanda S (2000) Hsp27 functions as a negative regulator of cytochrome c-dependent activation of procaspase-3. Oncogene 19:1975-1981.

Papadopoulos MC, Sun XY, Cao J, Mivechi NF, Giffard RG (1996) Over-expression of HSP-70 protects astrocytes from combined oxygen-glucose deprivation. Neuroreport 7:429-432.

Paul C, Manero F, Gonin S, Kretz-Remy C, Virot S, Arrigo AP (2002) Hsp27 as a negative regulator of cytochrome C release. Mol Cell Biol 22:816-834.

Peng H, Huang Y, Duan Z, Erdmann N, Xu D, Herek S, Zheng J (2005) Cellular IAP1 regulates TRAIL-induced apoptosis in human fetal cortical neural progenitor cells. J Neurosci Res 82:295-305.

Perng MD, Cairns L, van den IP, Prescott A, Hutcheson AM, Quinlan RA (1999) Intermediate filament interactions can be altered by HSP27 and alphaB-crystallin. J Cell Sci 112 (Pt 13):2099-2112.

Plumier JCL, Armstrong JN, Landry J, Babity JM, Robertson HA, Currie RW (1996) Expression of the 27,000 mol wt heat shock protein following kainic acid-induced status epilepticus in the rat. Neuroscience 75:849-856.

Renolleau S, Benjelloun N, Ben-Ari Y, Charriaut-Marlangue C (1997) Regulation of apoptosis-associated proteins in cell death following transient focal ischemia in rat pups. Apoptosis 2:368-376.

Robertson GS, Crocker SJ, Nicholson DW, Schulz JB (2000) Neuroprotection by the inhibition of apoptosis. Brain Pathol 10:283-292.

Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997) The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. Embo J 16:6914-6925.

Rubio E, Valenciano AI, Segundo C, Sanchez N, de Pablo F, de la Rosa EJ (2002) Programmed cell death in the neurulating embryo is prevented by the chaperone heat shock cognate 70. Eur J Neurosci 15:1646-1654.

Sanz O, Acarin L, Gonzalez B, Castellano B (2001) Expression of 27 kDa heat shock protein (Hsp27) in immature rat brain after a cortical aspiration lesion. Glia 36:259-270.

Sharp F, Kinouchi H, Koistinaho J, Chan PH, Sagar SM (1993) HSP70 heat shock gene regulation during ischemia. Stroke 24 (suppl I):I72-I75.

Sharp FR, Massa SM, Swanson RA (1999) Heat-shock protein protection. Trends Neurosci 22:97-99.

Sharp FR, Lowenstein D, Simon R, Hisanaga K (1991) Heat shock protein HSP72 induction in cortical and striatal astrocytes and neurons following infarction. J Cereb Blood Flow Metab 11:621-627.

Shin S, Sung BJ, Cho YS, Kim HJ, Ha NC, Hwang JI, Chung CW, Jung YK, Oh BH (2001) An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. Biochemistry 40:1117-1123.

Solaroglu I, Tsubokawa T, Cahill J, Zhang JH (2006) Anti-apoptotic effect of granulocyte-colony stimulating factor after focal cerebral ischemia in the rat. Neuroscience 143:965-974.

Sreedhar AS, Csermely P (2004) Heat shock proteins in the regulation of apoptosis: new strategies in tumor therapy: a comprehensive review. Pharmacol Ther 101:227-257.

Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T, Reed JC (1998) IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res 58:5315-5320.

Vassina EM, Yousefi S, Simon D, Zwicky C, Conus S, Simon HU (2006) cIAP-2 and survivin contribute to cytokine-mediated delayed eosinophil apoptosis. Eur J Immunol 36:1975-1984.

Vegran F, Boidot R, Oudin C, Riedinger JM, Lizard-Nacol S (2005) [Implication of alternative splice transcripts of caspase-3 and survivin in chemoresistance]. Bull Cancer 92:219-226.

Villapol S, Acarin L, Faiz M, Castellano B, onzalez B (2007) Distinct spatial and temporal activation of caspase pathways in neurons and glial cells after excitotoxic damage to the immature rat brain. Journal of Neuroscience Research.

Voss OH, Batra S, Kolattukudy SJ, Gonzalez-Mejia ME, Smith JB, Doseff AI (2007) Binding of caspase-3 prodomain to heat shock protein 27 regulates monocyte apoptosis by inhibiting caspase-3 proteolytic activation. J Biol Chem.

Xu L, Giffard RG (1997) HSP70 protects murine astrocytes from glucose deprivation injury. Neurosci Lett 224:9-12.

Yenari MA, Liu J, Zheng Z, Vexler ZS, Lee JE, Giffard RG (2005) Antiapoptotic and anti-inflammatory mechanisms of heat-shock protein protection. Ann N Y Acad Sci 1053:74-83.

4. SUMMARY OF RESULTS AND GENERAL DISCUSSION

4.1. NEURONAL CELLS

4.1.1. Apoptotic cell death and caspase-3 activation.

After a CNS acute injury early necrotic cell death is known to take place and is followed at later survival times by a delayed apoptotic death. Specifically, after excitotoxic damage to the immature brain, neuronal cell death has been demonstrated to occur as a necrosis/apoptosis continuum with progressive morphological characteristics [329]. Technically, it should be noted that the sensitivity and specificity of experimental methods might determine whether apoptosis is detected [283, 330, 331]. In this thesis, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) technique was used, which identifies 3´-OH end of DNA-strand breaks as a marker of DNA damage or repair. TUNEL staining was performed in order to look for evidences of DNA breakdown, one of the main features of apoptosis [283, 332]. DNA fragmentation is subject to problems of sensitivity, but evidence of caspase activation and demonstration of apoptotic bodies by DAPI staining or by Hoechst 33258 staining that allow visualization of nuclear chromatin clumping are considered a complementary method for demonstrating the presence of apoptotic features. Article I shows that following an excitotoxic lesion in the immature cortex the majority of cells undergoing apoptotic cell death were identified as neurons in the damaged cortex, hippocampus, and in the penumbra cortical area from 10 to 72 hours post-lesion (hpl), when DNA fragmentation decreases. Neuronal apoptosis occurred simultaneously was coexistent with cleaved caspase-3 staining observed at early times post-lesion in the neurodegenerative area, specifically in the medial cortex, lesion core, lesion border, and hippocampus, and a significant increase in caspase-3 enzymatic activity in the lesioned hemisphere. For the majority of the examined brain regions, neuronal caspase-3 immunoreactivity was clearly concentrated in the

nucleus of the cell and sometimes in soma and axonal prolongations. A nuclear localization of activated caspase-3 suggests the association of this protease with specific nuclear structures, as cleaved caspase-3 is the principal responsible for the cleavage of proteases that produces damage and destruction of nuclear DNA. These results are in agreement with other reports which have described neuronal apoptosis after injury in the neonatal rat brain [53, 320, 333-340].

4.1.2. Caspase-9 and caspase-8 activation in neurons in cortical damaged areas.

Caspase-3 neuronal activation occurs together with activation of the mitochondrial pathway as shown by an increase in the levels of active caspase-9 (article II). The peak of caspase-9 activation was at 10 hpl, and was followed by the principal increase in caspase-3 in the neurons and the presence of TUNEL + cells in most neurodegenerating areas at 24 hpl. The correlation of caspase-3 with caspase-9 at 10 hpl was found to be especially significant, as the number of caspase-3 + cells were roughly equivalent to the amount of caspase-9 + cells, at a time when both caspases were mainly observed in neuronal cells (Figure 6). In contrast, caspase-8 activation in the lesion core was strongly dependent on cortical layers specificity. At early times, the most significant expression was in layers I, II and III, where the lesion core is located, and after 24 hpl, caspase-8 expression was most important in layers V-VI. In these layers, caspase-8 was sometimes seen to colocalize with caspase-3, but not as significantly as the caspase-3/caspase-9 correlation, and only during the peak of both caspases, implying that the link between caspase-8 and caspase-3 was only transient and region specific. These results emphasize the involvement of the intrinsic pathway over the extrinsic pathway in neuronal death in the lesion core and medial cortex where the excitotoxic stimulus is greater and the mitochondrial damage predominates, however it should be mentioned that it might also coexist with other apoptotic pathways or with necrotic cell death. Moreover, in surrounding cortical areas and layer VI the mostly delayed involvement of caspase-8 activation was also observed, demonstrating that caspase-3 upstream activation pathways are also acting through death receptors. Of special interest is layer VI where subplate neurons reside. These neurons are highly studied after prenatal and early postnatal stroke because they are extremely susceptible to apoptotic cell death in the immature brain [321, 341, 342].

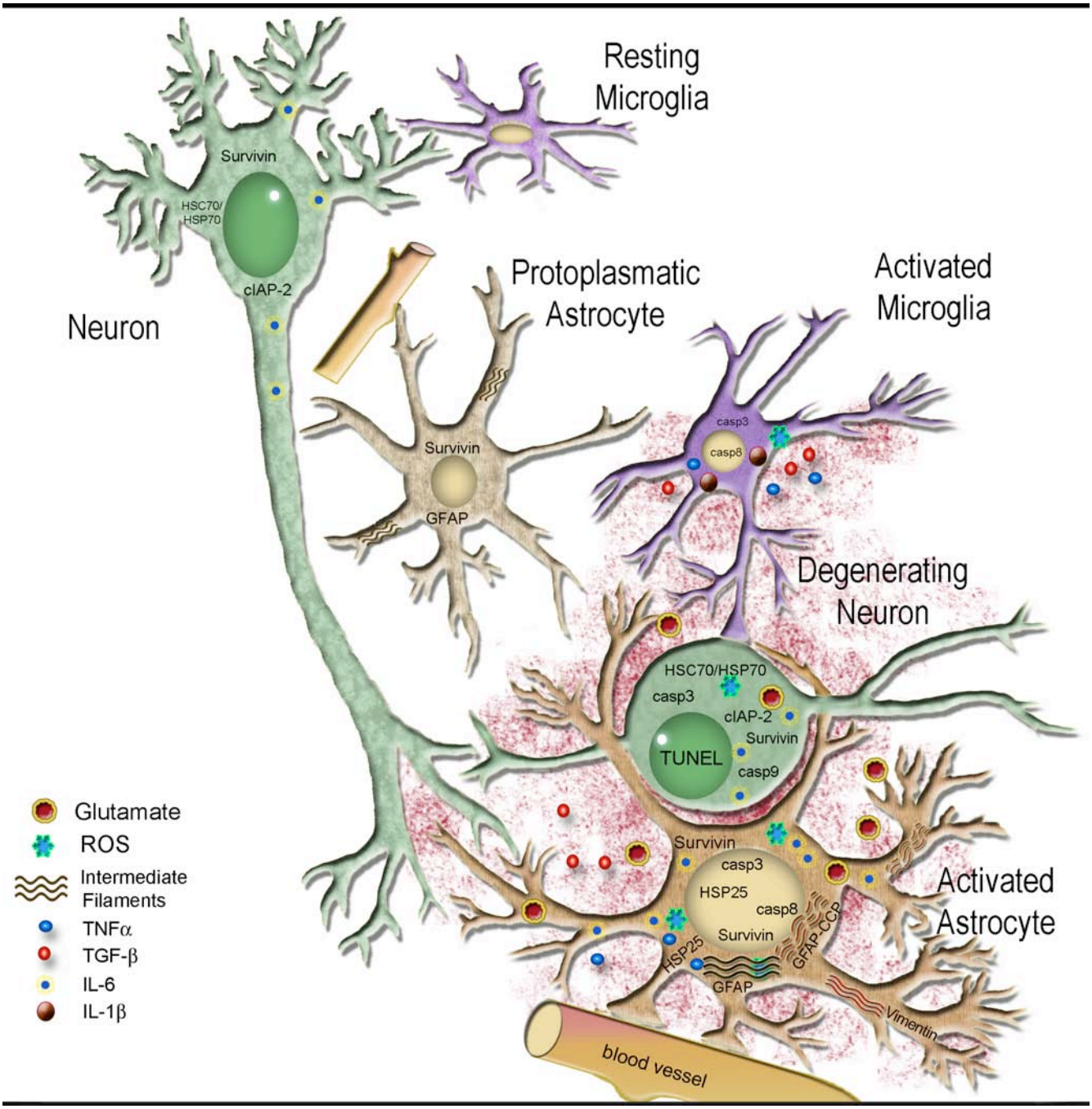


Figure 6. *Summary of results showing a schematic representation of apoptotic pathways and endogenous inhibitors in astrocytes, neurons and microglial cells after an excitotoxic damage in the immature rat brain. Degenerating neurons show activation of caspase -3 and -9 and undergo apoptotic cell death. Caspase-8 is expressed in the nucleus of some microglial cells. In addition, some antiapoptotic proteins cIAP-2 or HSC70/HSP70 are also expressed; reactive astrocytes show survivin and Hsp25 in the nucleus and cleaved caspase-3 in the absence of cell death, but with caspase-cleaved fragments of glial fibrillary acidic protein (GFAP-CCP) in the cytoskeleton.*

Notably, the number of TUNEL+ neurons seen in this experimental model always outnumbered those showing activation of caspase-3, caspase-9, caspase-8 or p53, suggesting an important contribution of other caspases or caspase-independent mechanisms of neuronal cell death, as has been suggested in other acute injury models both in adults and neonates (see [16, 343, 344] for review). One of the main candidates of caspase-independent mediators of cell death is the Apoptosis inducing factor (AIF), which is released form the mitochondria and reaches the nucleus where it can directly induce chromatin condensation and large scale DNA fragmentation [19, 345]. After NMDA excitotoxicity in cortical neurons *in vitro*, it is believed that AIF translocation, mitochondrial membrane depolarization and phosphatydil serine exposure on the cell surface occur prior to activation of the intrinsic pathway and caspase-3 cleavage [346], thus AIF would be faster in driving apoptotic cell death than caspase-3. The involvement of AIF and caspase-3 in neuronal apoptosis could establish a way for future approaches to reduce damage through combinatory methods, by inhibiting both caspase-dependent and caspase-independent apoptotic pathways as a therapeutic target for neuroprotection after acute injury.

4.1.3. Endogenous inhibitors in neurons.

All apoptotic mechanisms triggered in neurons after excitotoxic damage have been described to be counteracted by anti-apoptotic mechanisms. After studying diverse IAPs and HSPs, (article III) survivin expression, which is also seen in the contralateral hemisphere, was found in the soma of neurons located in the lesion core and lesion border and ipsilateral hippocampus. Several caspase-3 + neurons also expressed nuclear cytoplasmatic survivin but the majority of survivin + cells belonged to astroglials populations. Another studied IAP, cIAP-2, showed a cytoplasmatic staining in the lesion core, layer VI neurons and the lesion border. These IAPs may block apoptotic molecules and rescue neurons from death in regions where excitotoxic damage was not strong at specific times after injury, as it has been shown in other models of acute injury, both in the adult and postnatal brain [126, 133, 225, 347, 348]. Heat stress chaperones were also analyzed and expression in neurons was also found. HSP25/27 was expressed in the soma of neurons, restricted to the lesion border and hippocampus, but it was predominant in glia. However, HSC70/HSP70 was the main neuronal chaperone expressed both in the contralateral and ipsilateral hemispheres, principally affecting pyramidal neurons in layers I-IV and in the hippocampus. Even so, the neuroprotective role of this chaperone in this neonatal

excitotoxicity model is highly questioned, as it is rarely found to colocalize with caspase-3 in neurons. In another injury model, HSP72, a member of HSP70 subfamily, was also detected in the penumbra region expressed in the cytosol of surviving neurons playing a role in the inhibition of the mitochondrial pathway [343]. Apoptosis represents a target that potentially expands the therapeutic window for acute injury treatment because it appears to involve different mechanisms than necrosis and may occur in areas of milder ischemic injury, even days after the initial insult [349].

4.2. OLIGODENDROCYTES AND MICROGLIAL CELLS

In this thesis no considerable presence of cleaved caspase-3 or DNA fragmentation was found in microglia/macrophages (article I), and few cells showed nuclear caspase-8 and p53 in the nucleus (article II and Figure 6), mainly in the lesion core and layer VI. Activation of these molecules can be generally interpreted as playing a non-apoptotic role in microglial cells. In this sense, some studies have shown that caspase-8 may also be involved in other cellular functions like proliferation, differentiation, migration, or motility [26, 350-352], which could account for its activity in this cell type. With respect to oligodendrocytes, only a few TUNEL positive cells were seen (article I), they did not show evidence of cleaved caspase-3, sporadic caspase-9 and caspase-8 labeling in the cingulum and corpus callosum, and no p53 expression was detected. These results were unexpected as oligodendrocytes are vulnerable to be susceptible to excitotoxic damage [353-355], and periventricular leukomalacia [356] due to white matter damage is one of the leading pathological mechanisms of immature brain injury [357-359]. Studies in vivo have demonstrated that the oligodendrocytes show caspase-11 dependent-apoptotic death stimuli, induced by cytokines, nitric oxide, and oxidative stress [360]. Other works demonstrate that oligodendrocytes die through caspase-3 activation after perinatal hypoxia-ischemia [272]. Thus, a possibility is that the oligodendrocytes in our model are dying at very early times in white matter both by necrotic cell death, through other executor caspases and direct cleavage of nuclear substrates, or through autophagy as a possible protective mechanism in early stages of programmed cell death as was suggested in a transgenic model of dysmyelination [361].

4.3. ASTROCYTES

Apoptotic pathways at later times are glutamate-independent and may be triggered by other stimuli, including oxidative stress, acidosis, and calcium imbalance, which can mainly affect astrocytes. Elucidating whether astroglial cell death and activation of apoptotic pathways occurs after postnatal excitotoxicity was the main goal of this thesis, therefore its study has provided for the majority of results and discussion (see Figure 6).

4.3.1. Caspase-3 activation but reduced astrocytic death.

The first surprising result of this thesis was the finding that cleaved caspase-3 was present in astrocytes from 10 hpl, when double-labeled cleaved caspase-3/GFAP-positive astrocytes accounted for 35% of the total number of cleaved caspase-3 + cells. Later on, astrocytes accounted for the 70% of cleaved caspase-3 + cells. In contrast, the analysis of TUNEL/GFAP double-labeled sections displayed few astrocytes with TUNEL-positive nuclei. The number of astrocytes that died from apoptosis accounted from 10% of TUNEL + cells at 10 hpl, until less than 30% of the total number of TUNEL + cells identified at 7 dpl, when the total amount of TUNEL+ is very low (Article I). This finding opened several questions which generally constituted the aims of the thesis; to answer whether activation of caspase-3 upstream pathways were found in astrocytes, whether caspase-3 was inhibited in astrocytes downstream of its cleavage or whether caspase-3 might have alternative non-apoptotic roles in astrocytes.

4.3.1.1. Why and how can caspases show activation but not activity?

One possibility to explain how astrocytes show cleaved caspase-3 in the nucleus but not protease activity and cell death execution is that the amount of cleaved caspase-3 may not have been sufficient to cause DNA fragmentation. This phenomenon has been reported in cell cultures treated with staurosporine in a fascinating study that discovered that overexpression of a cDNA engineered for a chimeric caspase-3 protein, which resulted in active caspase-3 in the cells, did not induce any of the changes typical of apoptosis [362], and no visible cell morphological changes, due to low or insufficient caspase activity. In the results of this thesis, despite the appearance of a p17 fragment of

caspase-3 which is bound by the antibodies used, there was no evidence for enzymatic activity later than 24 hpl, when caspase-3 cleavage is mainly found in astrocytes (article I).

4.3.2. Upstream apoptotic pathways in astrocytes

4.3.2.1. The caspase cascade upstream of caspase-3 activation.

After cleaved caspase-3 was observed in astrocytes with no apparent morphology of apoptosis (article I), it was then studied whether activation of the main initiator caspases were activated at early times after excitotoxicity (article II). In astrocytes, the intrinsic mitochondrial pathway through caspase-9 activation was found to be much less important than in neurons, it was only observed in a few astrocytes in corpus callosum and lesion core at early times. Activation of the apoptotic extrinsic pathway through caspase-8 was more relevant, with a layer specific pattern, affecting astrocytes mainly in layer VI and the corpus callosum, and also important to note, caspase-8 labeling was also found in the nucleus of reactive GFAP astrocytes.

It should be noted that the active form of caspase-8 could not be detected by Western blot (data not shown) as has been published in another model of immature brain damage, using the same antibody [340], so we could assume that mainly the proform was detected. However, global results show that neither caspase-8 nor caspase-9 precede the activation of caspase-3 in astrocytes especially because caspase-3 is so widespread. Studies in ischemia have shown that both embryonic and mature astrocytes express a significant level of CD95 (Fas/APO-1) receptors, but only mature astrocytes are likely to undergo CD95-mediated apoptosis [313]. Other studies suggest that astroglial death can also implicate activation of the NF-κB inflammatory pathway instead of executing caspase-dependent cell death [85], although its relevance *in vivo* in acute models of damage is not clear. It should also be noted that other upstream caspases are thought to contribute to cell death via caspase-3-dependent pathways that are excluded from extrinsic and intrinsic pathways, and include mainly caspase-2, caspase-6, caspase-11 or caspase-12. In this regard, several in vitro studies have shown the importance of ER stress in astrocytes and caspase-12 activation as the main initiator caspase that cleaves caspase-3 [234] in this situation. Caspase-12 is activated by the release of Ca^{+2} from ER independently of caspase-8 or caspase-9 [363]. In this sense, astroglial caspase-12 is activated in acidosis conditions leading to

activation of caspase-3 [184, 229], but no expression of this caspase was found in the excitotoxic model used here (data not shown). Even though most of previous studies tried to show that caspase-12 might cleave effector caspases such as caspase-3 and caspase-7, other studies have shown a direct evidence of caspase-12 as a substrate for caspase-3 [364].

4.3.2.2. Could caspase-3 be autocleaved?

An interesting point is that in specific conditions and depending on the intensity of apoptotic stimulus, caspase-3 may suffer from auto-cleavage and not display sufficient catalytic capacity [218, 362]. This was described as an inefficient self-activation of caspase-3, and previous evidence suggests that adding a longer prodomain to a catalytic caspase domain generates a self-activating caspase. This should not exclude the possibility, however, that a small initial amount of caspase-3 activation is a positive feedback mechanism in which active caspase-3 cleaves procaspase-3, not unlike what occurs specifically in astrocytes under excitotoxic conditions where it is typically recovered as fully processed active protease. This auto-cleavage could partly of explain the absence of upstream apoptotic pathways in specific cell types like astrocytes.

4.3.2.3. Other pro-apoptotic molecules in astrocytes.

Another explanation for the absence of activation of extrinsic and intrinsic caspase upstream pathways could be the presence of other pro-apoptotic proteins that might play an important role in the interaction of the caspase cascade and end up in caspase-3 activation. Thus, caspase-3 activity has been linked with activation of the MAPKs, JNK, p38, p53, c-jun, and c-fos, albeit through the activation of intervening kinases. Pro-apoptotic protein expression may mainly reflect similar or different cellular events in different subtypes of nervous cells depending on the area of expression in the brain and the time-course after injury. In article II of this thesis the importance of the pattern of p53 expression in neurons and astrocytes was highlighted. Whereas p53 expression in neurons most likely signifies the activation of pro-apoptotic DNA damaged-induced apoptosis [365, 366]. P53 expression in astroglial cells might be a sign of differentiation, metabolic activation and cell cycle arrest, possibly to allow for repair of damaged DNA [36, 365]. However, it should be noted that caspase-3 could be activated by p53-mediated activation of caspase-8 and caspase-9. Even so, in a study on cisplatin-induced renal cells apoptosis it was seen that 50% of apoptotic cell death is mediated by p53, and that p53 activates

caspase-3 independently of either caspase-8, caspase-9 or mitochondrial dysfunction [367]. However, in our model no colocalization between caspase-3 and p53 nuclear translocation was observed (article II).

4.3.3. Caspase-3 blocking: inhibitors of apoptosis, heat shock proteins and nitric oxide.

The resistance of astrocytes to caspase-3 downstream events and apoptotic death remains highly controversial. However, several hypothetical mechanisms have been proposed which might be the presence of strong endogenous inhibitors of cleaved caspase-3, such as the IAPs, HSPs, and/or other specific conditions like are discussed here.

4.3.3.1. Why is caspase-3 activated but does not have apoptotic target molecules in astrocytes? Different ways to block caspase-3 activity.

Active caspase-3 is formed by two p17/p12 heterodimers and mainly induces the cell to undergo apoptosis by proteolyzing key cellular targets. The IAP family of proteins bind to activated caspase-3 and functionally sequester it or target it to the proteasome for degradation via C-terminal RING finger domains, which acts as an E3 ubiquitin ligase [368]. This family of anti-apoptotic proteins has several candidates; one of them is survivin [142], which has been shown to block the activity of caspase-3 once activated [123], and *in vivo* studies after TBI have shown that a large majority of astrocytes, but few neurons, express survivin both in the cytoplasm and the nucleus colocalize with active caspase-3 [142]. This is in agreement with what is described here after excitotoxic damage (article III), where cleaved caspase-3 is found in reactive astrocytes and an important contribution of survivin in blocking caspase-3 is demonstrated. Moreover, cIAP-2 is known to monoubiquitinate executioner caspases, but this modification is not normally associated with proteasome targeting and degradation as it might sequester active caspases from death substrates [369]. In this thesis it is described how astrocytes occasionally express nuclear cIAP-2 at later times in the corpus callosum and glial scar in cortical regions (article III). However, recent attention has focused on heat shock proteins as regulators of cell death and survival. Hence, the molecular mechanism by which HSPs family members inhibit the proteolytic maturation of caspase-3 is likely to be different from IAP family members. HSP27 may be inhibiting death receptor upstream of cleaved caspase-3; with an inhibition of both mitochondrial and

death receptor apoptotic pathways [143, 162, 370, 371]. HSP27 also binds to intermediate filaments such as GFAP to manage the intermediate filament network in astrocytes [372]. As HSP25/27 was expressed in reactive astrocytes, both in cytoplasm and nucleus, colocalizing with cleaved caspase-3, it is suggested that this chaperone might provide a checkpoint in the regulation of apoptosis by interaction with caspase-3. Finally, although presence of HSC70/HSP70 in astrocytes after neonatal excitotoxicity was not found (article III), other works have shown that HSP70 can block both apoptotic and necrotic astroglial death, and it is considered an especially interesting target for anti-ischemic therapy [193, 227, 267, 373] as it has been suggested that HSPs could contribute to astroglial survival when facing excitotoxicity and hypoxia-ischemia.

In addition, other endogenous caspase-3 modulating mechanisms which have not been explored here could potentially be expressed in astrocytes. Phosphoprotein enriched in astrocytes-15 kDa (PEA-15) is a protein that acts as a caspase-8 inhibitor in astrocytes [223, 374] and inhibits both apoptosis and proliferation in normal damaged astrocytes, as well as in gliomas [375]. PEA-15 is capable of protecting reactive astrocytes from apoptosis in hippocampus and corpus callosum using a model of astrogliosis occurring along motor neurons degeneration [374]. In addition to elucidating the reasons behind which the astrocytes express natural inhibitors of caspase-3, it will be necessary to know if these inhibitors are being released by the astrocytes at some time determined by the evolution of the cerebral lesion. Also, it is intriguing to better understand the role that astrocytes play in neighboring cell survival when releasing caspase-3 inhibiting molecules, and its upstream pathways. In function of this working hypothesis, astrocytes could play a fundamental role in the tolerance to damage and the maintenance of its survival, crucial for neuroprotection.

4.3.3.2. Putative role of NO in caspase-3 inhibition in astroglial cells.

After excitotoxic damage, reactive astrocytes show nitration of cytoplasmatic proteins and lack DNA fragmentation, but on the other hand, have cleaved caspase-3 in the nucleus ([322] and article I). NMDA receptors drive Ca^{2+} influx, which in turn activates the predominant NOS [376]. Activation of NOS generates NO, which leads to the formation of peroxynitrite [273], a potent oxidant that is formed by the rapid reaction of NO with superoxide anion radicals, predominating over the scavenging of ROS by superoxide dismutase. Nitration is the main footprint of peroxynitrite formation and induces

dysfunction of different proteins [377]. In this sense, S-nitrosylation of caspase-3, caspase-9, ASK1, and JNK block their activity and inhibit apoptosis, whereas S-nitrosylation of metalloproteinases, IKKb and NF-κB promotes cell death (for review [378]). NO supplied by exogenous NO donors serves in vivo as an anti-apoptotic regulator of caspase activity via S-nitrosylation of the Cys-163 residue of caspase-3 [379] [380], but controversial studies in culture have demonstrated that peroxynitrite (ONOO-) donor SIN-1 also induces apoptosis of astrocytes [220]. In view of the ambivalent capabilities of NO to act either in a pro-apoptotic or in an anti-apoptotic fashion depending on cell type and NO dosage, a complex spectrum of NO-mediated control of apoptosis is conceivable. In this sense, if a function of NO in the inhibition of astroglial caspase-3 is taken into account, the presence of nitrated species in reactive astrocytes [322] could be associated to the inhibition of caspase-3 and the NO induced S-nitrosylation of the cleaved fragment of caspase-3 which ultimately prevents the ability to recognize its substrates and could provide an antiapoptotic strategy.

4.3.4. Other roles for cleaved caspase-3

Previous reports have suggested that caspase-3 is capable of regulating non-apoptotic functions in certain cell types [103, 381-386]. In accordance with these suggestions, several functions of caspase-3 have been exposed.

4.3.4.1. Caspase-3 and cytoskeletal remodeling.

One of the possible non-apoptotic roles of caspase-3 in astrocytes is the cleavage of intermediate filaments, such as GFAP or vimentin, as first suggested by the group of T. Rohn, showing that astrogliosis can be associated to cleavage of filaments by caspases [113]. This study involving Alzheimer´s disease dementia demonstrated that caspase-mediated cleavage of GFAP and the activation of caspase-3 do not imply that astrocytes are dying through apoptosis as the cleavage of GFAP by caspases reflects the turnover of GFAP in reactive astrocytes, rather than over apoptosis [113]. However, in some astrocytes, this prolonged activation may induce apoptosis. The death of these reactive astrocytes can directly and/or indirectly affect functions and survival of the neighboring neurons [205]. In this thesis, astrocytes showing nuclear caspase-3 and intermediate filaments that were cleaved by caspase-3 were described (article I). It is possible that a "subapoptotic" activation of

caspase-3 serves one or more important functions, such as mediating cytoskeletal remodeling. It has been described that active caspase-3 initially appears in the cytoplasm at early stages of apoptosis and later in the nucleus [387]. This observation suggests the possibility that the enzymatic action of caspase-3 is first focused in the damage and cleavage of GFAP in the cytosol, involved in the process of cytoeskeletal remodeling and astrogliosis, and later after injury when its catalytic activity has decreased, it translocates to the nucleus, playing an unknown function or with a major susceptibility to be inhibited by endogenous inhibitors. This hypothesis would correlate with studies where it was seen that the catalytic activity of caspases can be graded to process the cleavage of specific substrates [388]. On the other hand, remodeling of intermediate filaments of reactive astrocytes can interrupt or cleave caspase-3 apoptotic substrates and can subsequently induce the translocation of caspase-3 to the nucleus where it does not have sufficient catalytic activity to cleave the habitual apoptotic substrates.

4.3.4.2. Nuclear caspase-3 and proliferation.

The activation of caspase-3 in the nucleus has become almost synonymous with apoptosis as active caspase-3 is principally responsible for the cleavage of nuclear proteases that produces damage and destruction to nuclear DNA, mainly PARP [263]. However, evidence has accumulated that activated caspase-3 is involved in a number of non-apoptotic processes participating in the cell cycle progression, proliferation and preconditioning [105]. BrdU (5-bromodeoxyuridine) is currently the most commonly used DNA replication marker which becomes incorporated into proliferating cell nuclei during S-phase. When the role of caspase-3 in astroglial proliferation was analyzed through the use of BrdU (article I) no double labeling of BrdU with cleaved caspase-3 was found, implying that astroglial cleaved caspase-3 are not necessary associated to proliferative role. Similarly, other studies have demonstrated constitutive, non-apoptotic activation of caspase-3 in the nuclei of Bergmann glia and a subpopulation of astrocytes in the cerebellar cortex, hippocampus, and spinal cord of rats of different strains of both sexes [389], suggesting that some nuclear substrates cleaved by caspase-3 can potentially be adaptive cellular responses and anti-apoptotic processes. Finally, another explanation could be that activated caspase-3 may be associated with specific nuclear structures, preventing access of activated caspase-3 to the normal protein substrates that lead towards apoptosis [390].

4.3.4.3. Glutamate transporters are also caspase targets.

Clearance of glutamate from the extracellular space at the synapse is necessary for normal neuronal signalling, and is primarily accomplished by Na^+ dependent transporters in astrocytes. Glial cells possess the glutamate transporters GLAST and GLT-1 (EAAT1, EAAT2) to remove extracellular glutamate, which allows for neuroprotection. This is in agreement with previous reports where the use of antisense oligonucleotides and subsequently inhibition of these transporters can induce the expansion of damage [391]. Interestingly, subpopulations of astrocytes that have GLAST receptors, such as the Bergmann glial cells, show constituently active caspase-3 without showing apoptotic morphology [389]. In relation to this, caspase-3 has been shown to activate an intermediate filaments protein named RhoA (a key protein involved in cytoskeleton regulation modulating neurogenesis and neural plasticity), that is thought to be linked to these receptors supporting its glutamate transport activity and favoring post-injury recovery, triggering anti-apoptotic mechanisms. In addition, in other cell types, RhoA directly interacts with fragmented vimentin filaments and plays an importance role in its remodeling. Therefore, it could be hypothesized that astroglial caspase-3 could be also implicated through RhoA in modulation of the glutamate transporters [259]. For this it would be necessary to first verify that RhoA can cleave the glutamate transporters of certain glial subpopulations (Glut-1, GLAST) and that caspase-3 has a preferred place of cleavage in RhoA and other filaments, such as Vimentin or GFAP.

5. CONCLUSIONS

General conclusion

The general conclusion of this study is that following excitotoxic damage to the immature cortex, astrocytes are highly resistant to DNA fragmentation, although cleaved caspase-3 is present in response to stimulus and could be responsible for cytoskeletal remodeling occurring in gliosis. In addition, astrocytes show expression of HSP25/27, a member of the heat shock protein family, and survivin, an inhibitor apoptosis protein, which can contribute to the blockade of astroglial caspase-3 in this animal model.

Specific conclusions

I. Neuronal cleaved caspase-3 is mainly associated with apoptotic cell death at early times post-injury.

II. Cleaved caspase-3 is mainly observed in astroglial nuclei and is not associated with TUNEL-labeling and apoptotic morphology.

III. The intrinsic pathway, through caspase-9, is mainly activated in neurons in cortical and hippocampal regions, but also in a few astrocytes in the corpus callosum.

IV. The extrinsic pathway through caspase-8 is activated in some cortical neurons but is layer specific. This pathway is also activated in some astrocytes and microglia.

V. Cleaved caspase-3 is not associated with proliferation correlates with caspase-cleaved GFAP filaments.

VI. Survivin and HSP25/27 are highly expressed in astrocytes and colocalize with caspase-3, however HSC70/HSP70 and cIAP-2 are mainly expressed in neurons and rarely colocalize with caspase-3.

6. ACKNOWLEDGMENTS

Agradecementos e Adicatoria

Aproveito este espazo irrepetible que me brinda o final desta tese para facer unha reflexión no meu sendeiro da investigación e render unha merecida homenaxe a todas aquelas persoas que me me axudaron neste traxecto. Transcurridos estes cuatro anos e medio, pasei etapas difíciles e duras, anque sempre gardando equilibrio con grandes satisfaccións, e en definitiva coma compensación todas estas circunstancias no seu conxunto acadaron fortalecerme.

A Bernardo y a Berta, os agradezco que me hayáis dado la posibilidad de hacer el doctorado en vuestro grupo y por seleccionarme para la beca predoctoral del departamento. A todos mis compañeros de laboratorio que fueron cómplices conmigo del periplo en la burbuja histológica, me ha encantado compartir y haber tenido una convivencia ejemplar con todos vosotros durante estes años, a todas/os; Dani, Oscar, Carol, Maria, Pau, Hugo, Bea, Montse, Mar, Lola, Miguel, Felix, Joaquín y a Valentín, moltes gràcies. And especially to Maryam, for your special friendship and for giving me your support in the lab when I needed it most. I will never forget the macroimmunos, the injections, the lesions we performed together, and the regular sprints to the FGC full of laughter we grew accustomed to after late nights working in the lab. I hope our paths cross again in the near future.

El meu immens reconeixement per a la meva directora de tesi, Laia Acarin, per tot el seu suport i per tot el temps que m'ha brindat. Em va ensenyar a escriure els papers, a muntar minuciosament les figures i em va convèncer que de moltes de les inseguretats que apareixen en l'etapa del doctorates poden solucionar seguint endavant i apostant amb empeny micant cap a endavant. T'agraeixo que m'hagis aconseguit el contracte de Professora Associada en aquesta etapa final de tesi, però sobretot per haver barallat per mi per la meva tesi. En definitiva sense la teva ajuda no hagués arribat fins a aquí. Petons per al Pau, Ton i la nena que vindrá. Des de París o Estats Units, seguirem en contacte.

Síntome moi recompensada pola Universitat Autònoma de Barcelona polo soporte que me prestou ó longo deste tempo de acollida. En particular, agradecer a eficacia do personal administrativo do rectorado que financiou as miñas duas estadas en París, e a asistencia ós congresos de Budapest e Chania. Tamén ás Marie Curie Actions por concederme as Fellowships para as Euroconferencias de Apoptosis.

Al resto de los colegas de la Autònoma, en especial a la gente del Departamento de Bioquímica y del Institut de Neurociències, a Eli Sanz por ayudarme con el Kit de actividad, a todo el clan de los apoptóticos; Rut, Montse, David, Nahuai, Alfredo, Bruna, Jose ... a Gema, al equipo de basket de los viernes en el SAF; a Enrique Claro por mi primer proyecto de investigación que me dió el DEA; a las chicas del IBB, Mariela, Paula, Judith y Marian por el intercambio de anticuerpos y los wbs; a Mónica por el soporte de tantas horas en el Confocal; a los vecinos de Psico; y a quellos aqueles investigadores jóvenes y valientes que han formado sus grupos aquí después de años en el extranjero, como a Carlos Saura o a Elena Galea, gracias por las charlas en los Ferrocarrils Catalans cargadas para mi de motivación ...

Ós autores dos papers que me resolveron dúbidas a través do fluxo de emails. Ás colaboracións con Troy R. e con Hervé Ch. que me enviaron anticorpos gratuítamente. E coma sempre, quedará alguén sen citar e que tamén contribuiu a que este traballo saíse a diante, mando un saúdo gremial para todos eles.

Non esquecerei tantas horas de docencia en Medicina, recordarei con nostalxia ós estudiantes que participaron nas tardes e tardes de clases enriba do microscopio, especialmente a todos os galegos, fillos e netos, e ós portugueses que acederon a que lles ensinara na miña lingua, para eles "o portugues mal falado", o que me permitiu estar máis preto da miña terra. E coma tamén existe traballo de investigación fora do laboratorio, un recordo para toda aquela xente coa que colaborei en Precarios e D-Reserca, por demostrarme que con pequenas loitas pódense acelerar os avances carreira científica neste país.

Vivir en Barcelona foi realmente un privilexio, disfrutar da cidade esperta, da arte co mar ó fondo da Rambla, do orgullo da lingua catalá. Sempre me lembrarei coma un lugar de cadros multicores, con toda a súa grandiosidade en construcción, amistosa coa xente de fora, fachendosa do seu, coma a gran cidade europea dos Barrios enxebres e acolledores. Para todos os amigos que nos levamos de Barcelona, cos que festexamos con pulpadas, ceas, salidas e festas, ...Xavier e Joana, Michaela e Jaime, Bea e Sergi, María, Jordi, Lucía e Jose Manuel, Benjamín e Jessica, ... Tamén a tantos colectivos culturais establecidos en Catalunya, coma ó Foro Cívico Galego ou ó Centro Galego, e a tódolos galegos que me atopei, que despois de décadas de emigración seguen a falar a sua lingua con grande dignidade.

E voltando ós meus comenzos, non quixera esquecerme de mostrar a miña gratitude a toda a xente coa que traballei en Santiago de Compostela durante a carreira, dende Farmacia onde empecei a xogar nun laboratorio de verdade por primeira vez, ata que cheguei a Fisio en Mediciña, onde tiven miñas primeiras celuliñas para transfectar Ghrelin e Pit. Agradézcolle a Clara que confiase en min decíndome que valía para isto, e por saber transmitirme tanta paixón e entusiasmo polo traballo no laboratorio, que tiña que ser meticuloso, sen obviar ningunha variable e facendo uso do sentido común para disfrutar ó máximo da ciencia. E ás miñas amigas, Ruth e Antía, que tamén traballaron por amor á ciencia, coas que compartín tantas confidencias e aprendizaxes no laboratorio.

Une des étapes que je me suis plus senties fière durant estes des années de thèse a été celle de mon séjour de recherche à Paris avec Christiane Charriaut-Marlangue. À Christiane je l'ai trouvée très par ses articles, en accord avec ce que je voulais faire, avec tant de clarté et tellement intelligemment conçus. À l'été de du 2006, effectuez un séjour dans son laboratoire U702 de NPA à Paris. Ce que maravilló de Christiane a plus été de écouter toujours dire un enthousiaste "why not?" comme réponse à mes propositions, ce qui arrête mon a signifié une dose optimisme et anxiété pour travailler. Là j'ai connu compagnon Maria Splieguer, qui m'a gentimentgentillement enseigné les lésions d'ischemia, et elle m'a aidée à m'intégrer rapidement dans le laboratoire. Au reste des membres du groupe, Sebastián, à Sylvian, Pierre Louis, Catherine, Celine, Mariani … j'ai même eu également le privilège de recevoir quelques Conseils du grand enseignant, Constantino Sotelo. Va-t-il être un plaisir réellement passer une saison de postdoc avec Christiane, ai-je beaucoup de désirs d'apprendre, travailler dur et sortir des résultats significatifs pour publier dans de bons journals, why not?

A dedicatoria fundamental desta tesis indudablemente é para os meus pais que quero tanto, Fernando Villapol e Antía Salgado, por darme exemplo de sacrificio e loita incansable obrigándonos a Ito e a min a sintirnos eternamente agradecidos. Sento admiración por ver que forman un equipo xenial que lles permitiu alcanzar o máximo, por riba dos seus proxectos. Souperon darme a libertade de collelo timón da miña vida sempre co seu apoio incondicional e tan necesario. Xa vedes que os meus fantasiosos xogos de laboratorio no meu sitio do desván cando era pequena, estanse facendo verdadeiros a partir de agora. Ó meu irmanciño Ito, por ser o meu amigo leal e para sempre, cómprice da miña infancia, e para Ana por aguantar a meu irmao. Ós meus avós, e á miña tataiña por quererme tanto dende que nacín.

To all of my family in USA, to Kay, Terry, Kurt, and Grandma Jessie, ... for your warm reception when we first met and always making me feel at home. And especially to my brother-in-law Terry II, for your logic and sensibility during our various philosophical discussions, and for not being ¨boring¨. Ós meus primos, Margarita e Manoliño, Pablo e David, Manuel e Juan, Noé, Moisés, César, Rocío, Nuria, Bea e Nicolás, xa sabedes que teredes unha prima lonxe á que visitar. A Paula, , a Xema, a Noemí, ... á Auruxeira.
A Meira, a Lugo, a Compostela, e a Bretoña sempre.

E para rematar, ó impulsor de que os meus soños se transformen día a día en feitos, a Todd James, o meu americano celta. Sacrificaches aquelas paredes brancas de Omaha onde xa estaba escrita a vida, para buscar algo máis. Só alguén que ten a capacidade para cambiar e adaptarse, poténciaselle a habilidade de avanzar e mellorar, e ti eres para min o mellor exemplo. Eres a fonte da miña ilusión e esperanza, a miña brúxula que sempre sabe onde están as mellores saídas, o motor que me impulsa a progresar e a lograr os nosos propósitos. Non sei que sería de min sen os teus consellos, a túa creatividade elocuente, transmítesme toda a confianza, calma, libertade, e un poder inmenso para poder esculpilos camiños da nosa vida ó noso xeito. Temos un libro azul aberto para debuxar a nosa apaixoada traxectoria perfumada de felicidade.

Barcelona, Setembro 2007

Sonia Villapol.

7. REFERENCES

1. Lockshin, R.A. and C.M. Williams, *Programmed Cell Death--I. Cytology Of Degeneration In The Intersegmental Muscles Of The Pernyi Silkmoth.* J Insect Physiol, 1965. **11**: p. 123-33.
2. Lockshin, R.A. and Z. Zakeri, *Programmed cell death and apoptosis: origins of the theory.* Nat Rev Mol Cell Biol, 2001. **2**(7): p. 545-50.
3. Kerr, J.F., A.H. Wyllie, and A.R. Currie, *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.* Br J Cancer, 1972. **26**(4): p. 239-57.
4. Ellis, H.M. and H.R. Horvitz, *Genetic control of programmed cell death in the nematode C. elegans.* Cell, 1986. **44**(6): p. 817-29.
5. Kanduc, D., et al., *Cell death: apoptosis versus necrosis (review).* Int J Oncol, 2002. **21**(1): p. 165-70.
6. Hengartner, M.O., *The biochemistry of apoptosis.* Nature, 2000. **407**(6805): p. 770-6.
7. Yuan, J. and B.A. Yankner, *Apoptosis in the nervous system.* Nature, 2000. **407**(6805): p. 802-9.
8. Earnshaw, W.C., L.M. Martins, and S.H. Kaufmann, *Mammalian caspases: structure, activation, substrates, and functions during apoptosis.* Annu Rev Biochem, 1999. **68**: p. 383-424.
9. Schulze-Osthoff, K., et al., *Apoptosis signaling by death receptors.* Eur J Biochem, 1998. **254**(3): p. 439-59.
10. Karki, P., et al., *Kinetic comparison of procaspase-3 and caspase-3.* Arch Biochem Biophys, 2005. **442**(1): p. 125-32.
11. Strasser, A., L. O'Connor, and V.M. Dixit, *Apoptosis signaling.* Annu Rev Biochem, 2000. **69**: p. 217-45.
12. Salvesen, G.S. and V.M. Dixit, *Caspase activation: the induced-proximity model.* Proc Natl Acad Sci U S A, 1999. **96**(20): p. 10964-7.
13. Lavrik, I.N., A. Golks, and P.H. Krammer, *Caspases: pharmacological manipulation of cell death.* J Clin Invest, 2005. **115**(10): p. 2665-72.
14. Budihardjo, I., et al., *Biochemical pathways of caspase activation during apoptosis.* Annu Rev Cell Dev Biol, 1999. **15**: p. 269-90.
15. Kroemer, G., L. Galluzzi, and C. Brenner, *Mitochondrial membrane permeabilization in cell death.* Physiol Rev, 2007. **87**(1): p. 99-163.
16. Love, S., *Apoptosis and brain ischaemia.* Prog Neuropsychopharmacol Biol Psychiatry, 2003. **27**(2): p. 267-82.
17. Green, D.R. and G. Kroemer, *The pathophysiology of mitochondrial cell death.* Science, 2004. **305**(5684): p. 626-9.
18. Costantini, P., et al., *Pre-processed caspase-9 contained in mitochondria participates in apoptosis.* Cell Death Differ, 2002. **9**(1): p. 82-8.
19. Susin, S.A., et al., *Two distinct pathways leading to nuclear apoptosis.* J Exp Med, 2000. **192**(4): p. 571-80.
20. Hirata, H., et al., *Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis.* J Exp Med, 1998. **187**(4): p. 587-600.
21. Rytomaa, M., L.M. Martins, and J. Downward, *Involvement of FADD and caspase-8 signalling in detachment-induced apoptosis.* Curr Biol, 1999. **9**(18): p. 1043-6.
22. Scaffidi, C., et al., *FLICE is predominantly expressed as two functionally active isoforms, caspase-8/a and caspase-8/b.* J Biol Chem, 1997. **272**(43): p. 26953-8.
23. Sohn, D., K. Schulze-Osthoff, and R.U. Janicke, *Caspase-8 can be activated by interchain proteolysis without receptor-triggered dimerization during drug-induced apoptosis.* J Biol Chem, 2005. **280**(7): p. 5267-73.
24. Li, T., et al., *Inhibition of caspase-8 attenuates neuronal death induced by limbic seizures in a cytochrome c-dependent and Smac/DIABLO-independent way.* Brain Res, 2006. **1098**(1): p. 204-11.
25. Velier, J.J., et al., *Caspase-8 and caspase-3 are expressed by different populations of cortical neurons undergoing delayed cell death after focal stroke in the rat.* J Neurosci, 1999. **19**(14): p. 5932-41.
26. Helfer, B., et al., *Caspase-8 promotes cell motility and calpain activity under nonapoptotic conditions.* Cancer Res, 2006. **66**(8): p. 4273-8.
27. Persad, R., et al., *Overexpression of caspase-3 in hepatocellular carcinomas.* Mod Pathol, 2004. **17**(7): p. 861-7.
28. Kurosu, K., M. Saeki, and Y. Kamisaki, *Formation of high molecular weight caspase-3 complex in neonatal rat brain.* Neurochem Int, 2004. **44**(4): p. 199-204.

29. Roth, K.A. and C. D'Sa, *Apoptosis and brain development.* Ment Retard Dev Disabil Res Rev, 2001. **7**(4): p. 261-6.
30. Le, D.A., et al., *Caspase activation and neuroprotection in caspase-3- deficient mice after in vivo cerebral ischemia and in vitro oxygen glucose deprivation.* Proc Natl Acad Sci U S A, 2002. **99**(23): p. 15188-93.
31. Kerr, L.E., et al., *Differential regulation of caspase-3 by pharmacological and developmental stimuli as demonstrated using humanised caspase-3 mice.* Apoptosis, 2004. **9**(6): p. 739-47.
32. Han, B.H., et al., *Selective, reversible caspase-3 inhibitor is neuroprotective and reveals distinct pathways of cell death after neonatal hypoxic-ischemic brain injury.* J Biol Chem, 2002. **277**(33): p. 30128-36.
33. Joly, L.M., et al., *Caspase inhibition after neonatal ischemia in the rat brain.* J Cereb Blood Flow Metab, 2004. **24**(1): p. 124-31.
34. Culmsee, C. and M.P. Mattson, *p53 in neuronal apoptosis.* Biochem Biophys Res Commun, 2005. **331**(3): p. 761-77.
35. Eizenberg, O., et al., *p53 plays a regulatory role in differentiation and apoptosis of central nervous system-associated cells.* Mol Cell Biol, 1996. **16**(9): p. 5178-85.
36. Galderisi, U., et al., *pRb2/p130 gene overexpression induces astrocyte differentiation.* Mol Cell Neurosci, 2001. **17**(3): p. 415-25.
37. Munoz-Pinedo, C., et al., *Different mitochondrial intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration.* Proc Natl Acad Sci U S A, 2006. **103**(31): p. 11573-8.
38. Siegelin, M.D., et al., *Regulation of XIAP and Smac/DIABLO in the rat hippocampus following transient forebrain ischemia.* Neurochem Int, 2005. **46**(1): p. 41-51.
39. Han, B.H., et al., *BDNF blocks caspase-3 activation in neonatal hypoxia-ischemia.* Neurobiol Dis, 2000. **7**(1): p. 38-53.
40. Kim, S.D., et al., *Identification of ASK1, MKK4, JNK, c-Jun, and caspase-3 as a signaling cascade involved in cadmium-induced neuronal cell apoptosis.* Biochem Biophys Res Commun, 2005. **328**(1): p. 326-34.
41. Modjtahedi, N., et al., *Apoptosis-inducing factor: vital and lethal.* Trends Cell Biol, 2006. **16**(5): p. 264-72.
42. Kim, J.T., et al., *Apoptosis-inducing factor (AIF) inhibits protein synthesis by interacting with the eukaryotic translation initiation factor 3 subunit p44 (eIF3g).* FEBS Lett, 2006. **580**(27): p. 6375-83.
43. Yuste, V.J., et al., *Cysteine protease inhibition prevents mitochondrial apoptosis-inducing factor (AIF) release.* Cell Death Differ, 2005. **12**(11): p. 1445-8.
44. Zhu, C., et al., *Nitrosylation precedes caspase-3 activation and translocation of apoptosis-inducing factor in neonatal rat cerebral hypoxia-ischaemia.* J Neurochem, 2004. **90**(2): p. 462-71.
45. Zhang, Q.G., et al., *Ischemic preconditioning negatively regulates plenty of SH3s-mixed lineage kinase 3-Rac1 complex and c-Jun N-terminal kinase 3 signaling via activation of Akt.* Neuroscience, 2006. **143**(2): p. 431-44.
46. Uchiyama, T., et al., *Role of Akt signaling in mitochondrial survival pathway triggered by hypoxic preconditioning.* Circulation, 2004. **109**(24): p. 3042-9.
47. Yamaguchi, A., et al., *Akt activation protects hippocampal neurons from apoptosis by inhibiting transcriptional activity of p53.* J Biol Chem, 2001. **276**(7): p. 5256-64.
48. Trencia, A., et al., *Protein kinase B/Akt binds and phosphorylates PED/PEA-15, stabilizing its antiapoptotic action.* Mol Cell Biol, 2003. **23**(13): p. 4511-21.
49. Luo, H.R., et al., *Akt as a mediator of cell death.* Proc Natl Acad Sci U S A, 2003. **100**(20): p. 11712-7.
50. Chen, X.Q., et al., *Inactivation of bad by site-specific phosphorylation: the checkpoint for ischemic astrocytes to initiate or resist apoptosis.* J Neurosci Res, 2005. **79**(6): p. 798-808.
51. Perez-Navarro, E., et al., *Brain-derived neurotrophic factor prevents changes in Bcl-2 family members and caspase-3 activation induced by excitotoxicity in the striatum.* J Neurochem, 2005. **92**(3): p. 678-91.
52. Vlodavsky, E., et al., *Evaluation of the apoptosis-related proteins of the BCL-2 family In the traumatic penumbra area of the rat model of cerebral contusion, treated by hyperbaric oxygen therapy: a quantitative immunohistochemical study.* Acta Neuropathol (Berl), 2005. **110**(2): p. 120-6.
53. Chen, Y., I. Ginis, and J.M. Hallenbeck, *The protective effect of ceramide in immature rat brain hypoxia-ischemia involves up-regulation of bcl-2 and reduction of TUNEL-positive cells.* J Cereb Blood Flow Metab, 2001. **21**(1): p. 34-40.
54. Rybnikova, E., et al., *The preconditioning modified neuronal expression of apoptosis-related proteins of Bcl-2 superfamily following severe hypobaric hypoxia in rats.* Brain Res, 2006. **1089**(1): p. 195-202.

55. Antignani, A. and R.J. Youle, *How do Bax and Bak lead to permeabilization of the outer mitochondrial membrane?* Curr Opin Cell Biol, 2006. **18**(6): p. 685-9.
56. Cato, A.C. and S. Mink, *BAG-1 family of cochaperones in the modulation of nuclear receptor action.* J Steroid Biochem Mol Biol, 2001. **78**(5): p. 379-88.
57. Liu, D., et al., *Activation of mitochondrial ATP-dependent potassium channels protects neurons against ischemia-induced death by a mechanism involving suppression of Bax translocation and cytochrome c release.* J Cereb Blood Flow Metab, 2002. **22**(4): p. 431-43.
58. Yung, H.W., et al., *Nitric oxide-induced cell death of cerebrocortical murine astrocytes is mediated through p53- and Bax-dependent pathways.* J Neurochem, 2004. **89**(4): p. 812-21.
59. Tung, T.C., et al., *Dual upregulation of Fas and Bax promotes alloreactive T cell apoptosis in IL-10 gene targeting of cardiac allografts.* Am J Physiol Heart Circ Physiol, 2003. **285**(3): p. H964-73.
60. Kohji, T. and Y. Matsumoto, *Coexpression of Fas/FasL and Bax on brain and infiltrating T cells in the central nervous system is closely associated with apoptotic cell death during autoimmune encephalomyelitis.* J Neuroimmunol, 2000. **106**(1-2): p. 165-71.
61. Gibson, M.E., et al., *BAX contributes to apoptotic-like death following neonatal hypoxia-ischemia: evidence for distinct apoptosis pathways.* Mol Med, 2001. **7**(9): p. 644-55.
62. Tilly, J.L., *Personal reflections on Stan Korsmeyer and his continuing impact on our work to delineate the role of Bax in female reproduction.* Cell Death Differ, 2006. **13**(8): p. 1296-8.
63. Polster, B.M., et al., *Postnatal brain development and neural cell differentiation modulate mitochondrial Bax and BH3 peptide-induced cytochrome c release.* Cell Death Differ, 2003. **10**(3): p. 365-70.
64. Reed, J.C., *Proapoptotic multidomain Bcl-2/Bax-family proteins: mechanisms, physiological roles, and therapeutic opportunities.* Cell Death Differ, 2006. **13**(8): p. 1378-86.
65. Yin, W., et al., *TAT-mediated delivery of Bcl-xL protein is neuroprotective against neonatal hypoxic-ischemic brain injury via inhibition of caspases and AIF.* Neurobiol Dis, 2006. **21**(2): p. 358-71.
66. Naumann, U., et al., *Alkylphosphocholine-induced glioma cell death is BCL-X(L)-sensitive, caspase-independent and characterized by massive cytoplasmic vacuole formation.* Cell Death Differ, 2004. **11**(12): p. 1326-41.
67. Franz, G., et al., *Temporal and spatial profile of Bid cleavage after experimental traumatic brain injury.* J Cereb Blood Flow Metab, 2002. **22**(8): p. 951-8.
68. Minami, M., et al., *Bcl-w expression is increased in brain regions affected by focal cerebral ischemia in the rat.* Neurosci Lett, 2000. **279**(3): p. 193-5.
69. Meller, R., et al., *Rapid degradation of Bim by the ubiquitin-proteasome pathway mediates short-term ischemic tolerance in cultured neurons.* J Biol Chem, 2006. **281**(11): p. 7429-36.
70. Wang, X., et al., *Activation of ERK1/2 after neonatal rat cerebral hypoxia-ischaemia.* J Neurochem, 2003. **86**(2): p. 351-62.
71. Jiang, Z., et al., *Apoptosis and activation of Erkl/2 and Akt in astrocytes postischemia.* Neurochem Res, 2003. **28**(6): p. 831-7.
72. Sur, R. and J.W. Ramos, *Vanishin is a novel ubiquitinylated death-effector domain protein that blocks ERK activation.* Biochem J, 2005. **387**(Pt 2): p. 315-24.
73. Jones, N.M. and M. Bergeron, *Hypoxia-induced ischemic tolerance in neonatal rat brain involves enhanced ERK1/2 signaling.* J Neurochem, 2004. **89**(1): p. 157-67.
74. Kuwabara, M., K. Takahashi, and O. Inanami, *Induction of apoptosis through the activation of SAPK/JNK followed by the expression of death receptor Fas in X-irradiated cells.* J Radiat Res (Tokyo), 2003. **44**(3): p. 203-9.
75. Xifro, X., et al., *NMDA blocks activation of JNK and mitochondrial apoptotic pathway induced by potassium deprivation in cerebellar granule cells.* J Biol Chem, 2005.
76. Sun, H.Y., et al., *Postconditioning attenuates cardiomyocyte apoptosis via inhibition of JNK and p38 mitogen-activated protein kinase signaling pathways.* Apoptosis, 2006. **11**(9): p. 1583-93.
77. Guan, Q.H., et al., *Neuroprotection against ischemic brain injury by a small peptide inhibitor of c-Jun N-terminal kinase (JNK) via nuclear and non-nuclear pathways.* Neuroscience, 2006. **139**(2): p. 609-27.
78. Xifro, X., et al., *N-methyl-D-aspartate blocks activation of JNK and mitochondrial apoptotic pathway induced by potassium deprivation in cerebellar granule cells.* J Biol Chem, 2006. **281**(10): p. 6801-12.

79. Kaushal, G.P., et al., *Regulation of caspase-3 and -9 activation in oxidant stress to RTE by forkhead transcription factors, Bcl-2 proteins, and MAP kinases.* Am J Physiol Renal Physiol, 2004. **287**(6): p. F1258-68.
80. Oh, H.L., et al., *Role of MAPK in ceramide-induced cell death in primary cultured astrocytes from mouse embryonic brain.* Neurotoxicology, 2005.
81. Ferrer, I., et al., *Phosphorylated mitogen-activated protein kinase (MAPK/ERK-P), protein kinase of 38 kDa (p38-P), stress-activated protein kinase (SAPK/JNK-P), and calcium/calmodulin-dependent kinase II (CaM kinase II) are differentially expressed in tau deposits in neurons and glial cells in tauopathies.* J Neural Transm, 2001. **108**(12): p. 1397-415.
82. Suzuki, Y., et al., *Ability of NMDA and non-NMDA receptor antagonists to inhibit cerebral ischemic damage in aged rats.* Brain Res, 2003. **964**(1): p. 116-20.
83. Yang, X., et al., *Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway.* Dev Biol, 2004. **269**(1): p. 81-94.
84. Natoli, G., et al., *Nuclear factor kB-independent cytoprotective pathways originating at tumor necrosis factor receptor-associated factor 2.* J Biol Chem, 1998. **273**(47): p. 31262-72.
85. Lemmers, B., et al., *Essential role for caspase-8 in toll-like receptors and NFkappa B signalling.* J Biol Chem, 2007.
86. Deacon, K., et al., *p38 Mitogen-activated protein kinase mediates cell death and p21-activated kinase mediates cell survival during chemotherapeutic drug-induced mitotic arrest.* Mol Biol Cell, 2003. **14**(5): p. 2071-87.
87. Araujo, H., et al., *Characterization of PEA-15, a major substrate for protein kinase C in astrocytes.* J Biol Chem, 1993. **268**(8): p. 5911-20.
88. Danziger, N., et al., *Cellular expression, developmental regulation, and phylogenic conservation of PEA-15, the astrocytic major phosphoprotein and protein kinase C substrate.* J Neurochem, 1995. **64**(3): p. 1016-25.
89. Estelles, A., et al., *The major astrocytic phosphoprotein PEA-15 is encoded by two mRNAs conserved on their full length in mouse and human.* J Biol Chem, 1996. **271**(25): p. 14800-6.
90. Condorelli, G., et al., *PED/PEA-15 gene controls glucose transport and is overexpressed in type 2 diabetes mellitus.* Embo J, 1998. **17**(14): p. 3858-66.
91. Estelles, A., C.A. Charlton, and H.M. Blau, *The phosphoprotein protein PEA-15 inhibits Fas- but increases TNF-R1-mediated caspase-8 activity and apoptosis.* Dev Biol, 1999. **216**(1): p. 16-28.
92. Cregan, S.P., et al., *Bax-dependent caspase-3 activation is a key determinant in p53-induced apoptosis in neurons.* J Neurosci, 1999. **19**(18): p. 7860-9.
93. Alonso, M., et al., *Flavopiridol induces apoptosis in glioma cell lines independent of retinoblastoma and p53 tumor suppressor pathway alterations by a caspase-independent pathway.* Mol Cancer Ther, 2003. **2**(2): p. 139-50.
94. Jin, S., et al., *Gadd45a contributes to p53 stabilization in response to DNA damage.* Oncogene, 2003. **22**(52): p. 8536-40.
95. Katakowski, M., et al., *Phosphoinositide 3-kinase promotes adult subventricular neuroblast migration after stroke.* J Neurosci Res, 2003. **74**(4): p. 494-501.
96. Hirai, K., et al., *PI3K inhibition in neonatal rat brain slices during and after hypoxia reduces phospho-Akt and increases cytosolic cytochrome c and apoptosis.* Brain Res Mol Brain Res, 2004. **124**(1): p. 51-61.
97. Essmann, F., et al., *Irradiation-induced translocation of p53 to mitochondria in the absence of apoptosis.* J Biol Chem, 2005. **280**(44): p. 37169-77.
98. Tenneti, L. and S.A. Lipton, *Involvement of activated caspase-3-like proteases in N-methyl-D-aspartate-induced apoptosis in cerebrocortical neurons.* J Neurochem, 2000. **74**(1): p. 134-42.
99. Gilliams-Francis, K.L., A.A. Quaye, and J.R. Naegele, *PARP cleavage, DNA fragmentation, and pyknosis during excitotoxin-induced neuronal death.* Exp Neurol, 2003. **184**(1): p. 359-72.
100. Horky, M., et al., *Segregation of nucleolar components coincides with caspase-3 activation in cisplatin-treated HeLa cells.* J Cell Sci, 2001. **114**(Pt 4): p. 663-70.
101. Doostzadeh-Cizeron, J., et al., *Apoptosis induced by the nuclear death domain protein p84N5 is inhibited by association with Rb protein.* Mol Biol Cell, 1999. **10**(10): p. 3251-61.
102. Utz, P.J. and P. Anderson, *Life and death decisions: regulation of apoptosis by proteolysis of signaling molecules.* Cell Death Differ, 2000. **7**(7): p. 589-602.
103. Launay, S., et al., *Vital functions for lethal caspases.* Oncogene, 2005. **24**(33): p. 5137-48.

104. Lamkanfi, M., et al., *Caspases in cell survival, proliferation and differentiation.* Cell Death Differ, 2007. **14**(1): p. 44-55.
105. Algeciras-Schimnich, A., B.C. Barnhart, and M.E. Peter, *Apoptosis-independent functions of killer caspases.* Curr Opin Cell Biol, 2002. **14**(6): p. 721-6.
106. Schwerk, C. and K. Schulze-Osthoff, *Non-apoptotic functions of caspases in cellular proliferation and differentiation.* Biochem Pharmacol, 2003. **66**(8): p. 1453-8.
107. Banasiak, K.J., Y. Xia, and G.G. Haddad, *Mechanisms underlying hypoxia-induced neuronal apoptosis.* Prog Neurobiol, 2000. **62**(3): p. 215-49.
108. McLaughlin, B., *The kinder side of killer proteases: caspase activation contributes to neuroprotection and CNS remodeling.* Apoptosis, 2004. **9**(2): p. 111-21.
109. Oliver, L. and F.M. Vallette, *The role of caspases in cell death and differentiation.* Drug Resist Updat, 2005. **8**(3): p. 163-70.
110. Yan, X.X., et al., *Expression of active caspase-3 in mitotic and postmitotic cells of the rat forebrain.* J Comp Neurol, 2001. **433**(1): p. 4-22.
111. Mattson, M.P. and D. Liu, *Mitochondrial potassium channels and uncoupling proteins in synaptic plasticity and neuronal cell death.* Biochem Biophys Res Commun, 2003. **304**(3): p. 539-49.
112. Morishima, N., *Changes in nuclear morphology during apoptosis correlate with vimentin cleavage by different caspases located either upstream or downstream of Bcl-2 action.* Genes Cells, 1999. **4**(7): p. 401-14.
113. Mouser, P.E., et al., *Caspase-Mediated Cleavage of Glial Fibrillary Acidic Protein within Degenerating Astrocytes of the Alzheimer's Disease Brain.* Am J Pathol, 2006. **168**(3): p. 936-46.
114. McLaughlin, B., et al., *Caspase 3 activation is essential for neuroprotection in preconditioning.* Proc Natl Acad Sci U S A, 2003. **100**(2): p. 715-20.
115. Qi, S., et al., *Sublethal cerebral ischemia inhibits caspase-3 activation induced by subsequent prolonged ischemia in the C57Black/Crj6 strain mouse.* Neurosci Lett, 2001. **315**(3): p. 133-6.
116. Tanaka, H., et al., *Ischemic preconditioning: neuronal survival in the face of caspase-3 activation.* J Neurosci, 2004. **24**(11): p. 2750-9.
117. Levkau, B., et al., *Cleavage of p21Cip1/Waf1 and p27Kip1 mediates apoptosis in endothelial cells through activation of Cdk2: role of a caspase cascade.* Mol Cell, 1998. **1**(4): p. 553-63.
118. Kuan, C.Y., et al., *Hypoxia-ischemia induces DNA synthesis without cell proliferation in dying neurons in adult rodent brain.* J Neurosci, 2004. **24**(47): p. 10763-72.
119. Miura, M., et al., *A crucial role of caspase-3 in osteogenic differentiation of bone marrow stromal stem cells.* J Clin Invest, 2004. **114**(12): p. 1704-13.
120. Wang, Z., et al., *Cellular, biochemical, and genetic analysis of mechanism of small molecule IAP inhibitors.* J Biol Chem, 2004. **279**(46): p. 48168-76.
121. Nachmias, B., Y. Ashhab, and D. Ben-Yehuda, *The inhibitor of apoptosis protein family (IAPs): an emerging therapeutic target in cancer.* Semin Cancer Biol, 2004. **14**(4): p. 231-43.
122. Prunell, G.F. and C.M. Troy, *Balancing neuronal death.* J Neurosci Res, 2004. **78**(1): p. 1-6.
123. Shin, S., et al., *An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7.* Biochemistry, 2001. **40**(4): p. 1117-23.
124. Xu, D.G., et al., *Elevation of neuronal expression of NAIP reduces ischemic damage in the rat hippocampus.* Nat Med, 1997. **3**(9): p. 997-1004.
125. Conway, E.M., et al., *Survivin-dependent angiogenesis in ischemic brain: molecular mechanisms of hypoxia-induced up-regulation.* Am J Pathol, 2003. **163**(3): p. 935-46.
126. Trapp, T., et al., *Transgenic mice overexpressing XIAP in neurons show better outcome after transient cerebral ischemia.* Mol Cell Neurosci, 2003. **23**(2): p. 302-13.
127. Johnson, E.A., et al., *Cell-specific DNA fragmentation may be attenuated by a survivin-dependent mechanism after traumatic brain injury in rats.* Exp Brain Res, 2005. **167**(1): p. 17-26.
128. Jiang, Y., et al., *Essential role for survivin in early brain development.* J Neurosci, 2005. **25**(30): p. 6962-70.
129. Wang, X., et al., *X-linked inhibitor of apoptosis (XIAP) protein protects against caspase activation and tissue loss after neonatal hypoxia-ischemia.* Neurobiol Dis, 2004. **16**(1): p. 179-89.

130. Korhonen, L., et al., *Differential regulation of X-chromosome-linked inhibitor of apoptosis protein (XIAP) and caspase-3 by NMDA in developing hippocampal neurons; involvement of the mitochondrial pathway in NMDA-mediated neuronal survival.* Exp Cell Res, 2004. **295**(2): p. 290-9.
131. Ishigaki, S., et al., *X-Linked inhibitor of apoptosis protein is involved in mutant SOD1-mediated neuronal degeneration.* J Neurochem, 2002. **82**(3): p. 576-84.
132. Korhonen, L., N. Belluardo, and D. Lindholm, *Regulation of X-chromosome-linked inhibitor of apoptosis protein in kainic acid-induced neuronal death in the rat hippocampus.* Mol Cell Neurosci, 2001. **17**(2): p. 364-72.
133. Robertson, G.S., et al., *Neuroprotection by the inhibition of apoptosis.* Brain Pathol, 2000. **10**(2): p. 283-92.
134. Keane, R.W., et al., *Apoptotic and antiapoptotic mechanisms after traumatic brain injury.* J Cereb Blood Flow Metab, 2001. **21**(10): p. 1189-98.
135. Peng, H., et al., *Cellular IAP1 regulates TRAIL-induced apoptosis in human fetal cortical neural progenitor cells.* J Neurosci Res, 2005. **82**(3): p. 295-305.
136. Simons, M., et al., *Adenovirus-mediated gene transfer of inhibitors of apoptosis protein delays apoptosis in cerebellar granule neurons.* J Neurochem, 1999. **72**(1): p. 292-301.
137. Suh, J., Y.A. Lee, and B.J. Gwag, *Induction and attenuation of neuronal apoptosis by proteasome inhibitors in murine cortical cell cultures.* J Neurochem, 2005. **95**(3): p. 684-94.
138. Mercer, E.A., et al., *NAIP interacts with hippocalcin and protects neurons against calcium-induced cell death through caspase-3-dependent and -independent pathways.* Embo J, 2000. **19**(14): p. 3597-607.
139. Lesne, S., et al., *Akt-dependent expression of NAIP-1 protects neurons against amyloid-{beta} toxicity.* J Biol Chem, 2005. **280**(26): p. 24941-7.
140. Korhonen, L., et al., *Hippocalcin protects against caspase-12-induced and age-dependent neuronal degeneration.* Mol Cell Neurosci, 2005. **28**(1): p. 85-95.
141. Marks, N. and M.J. Berg, *Recent advances on neuronal caspases in development and neurodegeneration.* Neurochem Int, 1999. **35**(3): p. 195-220.
142. Johnson, E.A., et al., *Cell-specific upregulation of survivin after experimental traumatic brain injury in rats.* J Neurotrauma, 2004. **21**(9): p. 1183-95.
143. Concannon, C.G., S. Orrenius, and A. Samali, *Hsp27 inhibits cytochrome c-mediated caspase activation by sequestering both pro-caspase-3 and cytochrome c.* Gene Expr, 2001. **9**(4-5): p. 195-201.
144. Wang, X., et al., *Prostaglandin A1 inhibits rotenone-induced apoptosis in SH-SY5Y cells.* J Neurochem, 2002. **83**(5): p. 1094-102.
145. Chiral, M., et al., *Effects of hypoxia on stress proteins in the piglet brain at birth.* Pediatr Res, 2004. **56**(5): p. 775-82.
146. Fauconneau, B., et al., *Induction of heat shock proteins (HSPs) by sodium arsenite in cultured astrocytes and reduction of hydrogen peroxide-induced cell death.* J Neurochem, 2002. **83**(6): p. 1338-48.
147. Sanz, O., et al., *Expression of 27 kDa heat shock protein (Hsp27) in immature rat brain after a cortical aspiration lesion.* Glia, 2001. **36**(3): p. 259-70.
148. Tezel, G. and M.B. Wax, *The mechanisms of hsp27 antibody-mediated apoptosis in retinal neuronal cells.* J Neurosci, 2000. **20**(10): p. 3552-62.
149. Acarin, L., et al., *Glial expression of small heat shock proteins following an excitotoxic lesion in the immature rat brain.* Glia, 2002. **38**(1): p. 1-14.
150. Adori, C., et al., *Damage of serotonergic axons and immunolocalization of Hsp27, Hsp72, and Hsp90 molecular chaperones after a single dose of MDMA administration in Dark Agouti rat: temporal, spatial, and cellular patterns.* J Comp Neurol, 2006. **497**(2): p. 251-69.
151. Currie, R.W., et al., *Benign focal ischemic preconditioning induces neuronal Hsp70 and prolonged astrogliosis with expression of Hsp27.* Brain Res, 2000. **863**(1-2): p. 169-81.
152. Kawamoto, Y., et al., *Accumulation of Hsc70 and Hsp70 in glial cytoplasmic inclusions in patients with multiple system atrophy.* Brain Res, 2007. **1136**(1): p. 219-27.
153. Liu, C., et al., *Ischemic preconditioning prevents protein aggregation after transient cerebral ischemia.* Neuroscience, 2005. **134**(1): p. 69-80.
154. Jaattela, M., et al., *Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases.* Embo J, 1998. **17**(21): p. 6124-34.

155. Lee, S.H., et al., *Effects of hsp70.1 gene knockout on the mitochondrial apoptotic pathway after focal cerebral ischemia.* Stroke, 2004. **35**(9): p. 2195-9.
156. Cizkova, D., et al., *Characterization of spinal HSP72 induction and development of ischemic tolerance after spinal ischemia in rats.* Exp Neurol, 2004. **185**(1): p. 97-108.
157. Gashegu, J., et al., *Correlation of Hsp110 expression with caspase-3 and -9 during apoptosis induced by in vivo embryonic exposition to retinoic acid or irradiation in early mouse craniofacial development.* Orthod Craniofac Res, 2006. **9**(2): p. 84-92.
158. Sreedhar, A.S. and P. Csermely, *Heat shock proteins in the regulation of apoptosis: new strategies in tumor therapy: a comprehensive review.* Pharmacol Ther, 2004. **101**(3): p. 227-57.
159. Kiang, J.G. and G.C. Tsokos, *Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology.* Pharmacol Ther, 1998. **80**(2): p. 183-201.
160. Omata, N., et al., *Region-specific induction of hypoxic tolerance by expression of stress proteins and antioxidant enzymes.* Neurol Sci, 2006. **27**(1): p. 74-7.
161. Arrigo, A.P., et al., *Hsp27 (HspB1) and alphaB-crystallin (HspB5) as therapeutic targets.* FEBS Lett, 2007.
162. Arya, R., M. Mallik, and S.C. Lakhotia, *Heat shock genes-integrating cell survival and death.* J Biosci, 2007. **32**(3): p. 595-610.
163. David, J.C., W.C. Boelens, and J.F. Grongnet, *Up-regulation of heat shock protein HSP 20 in the hippocampus as an early response to hypoxia of the newborn.* J Neurochem, 2006.
164. Concannon, C.G., A.M. Gorman, and A. Samali, *On the role of Hsp27 in regulating apoptosis.* Apoptosis, 2003. **8**(1): p. 61-70.
165. Valentim, L.M., et al., *Effects of global cerebral ischemia and preconditioning on heat shock protein 27 immunocontent and phosphorylation in rat hippocampus.* Neuroscience, 2001. **107**(1): p. 43-9.
166. Valentim, L.M., et al., *Changes in heat shock protein 27 phosphorylation and immunocontent in response to preconditioning to oxygen and glucose deprivation in organotypic hippocampal cultures.* Neuroscience, 2003. **118**(2): p. 379-86.
167. Gotoh, T., et al., *hsp70-DnaJ chaperone pair prevents nitric oxide- and CHOP-induced apoptosis by inhibiting translocation of Bax to mitochondria.* Cell Death Differ, 2004. **11**(4): p. 390-402.
168. Kim, S.W. and J.K. Lee, *NO-induced downregulation of HSP10 and HSP60 expression in the postischemic brain.* J Neurosci Res, 2007.
169. Samali, A., et al., *Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of jurkat cells.* Embo J, 1999. **18**(8): p. 2040-8.
170. Jiang, J., et al., *Structural basis of interdomain communication in the Hsc70 chaperone.* Mol Cell, 2005. **20**(4): p. 513-24.
171. Wang, Q., D.D. Mosser, and J. Bag, *Induction of HSP70 expression and recruitment of HSC70 and HSP70 in the nucleus reduce aggregation of a polyalanine expansion mutant of PABPN1 in HeLa cells.* Hum Mol Genet, 2005. **14**(23): p. 3673-84.
172. Tutar, Y., Y. Song, and D.C. Masison, *Primate chaperones Hsc70 (constitutive) and Hsp70 (induced) differ functionally in supporting growth and prion propagation in Saccharomyces cerevisiae.* Genetics, 2006. **172**(2): p. 851-61.
173. Steel, R., et al., *Hsp72 inhibits apoptosis upstream of the mitochondria and not through interactions with Apaf-1.* J Biol Chem, 2004. **279**(49): p. 51490-9.
174. Clemons, N.J., et al., *Hsp72 inhibits Fas-mediated apoptosis upstream of the mitochondria in type II cells.* J Biol Chem, 2005. **280**(10): p. 9005-12.
175. Manabe, Y., et al., *Expressions of caspase-3, Tunel, and Hsp72 immunoreactivities in cultured spinal cord neurons of rat after exposure to glutamate, nitric oxide, or peroxynitrite.* Neurotox Res, 2001. **3**(3): p. 281-9.
176. Malusecka, E., et al., *Immunohistochemical detection of the inducible heat shock protein hsp70: a methodological study.* J Histochem Cytochem, 2006. **54**(2): p. 183-90.
177. Robinson, M.B., et al., *Extracellular heat shock protein 70: a critical component for motoneuron survival.* J Neurosci, 2005. **25**(42): p. 9735-45.
178. Lai, Y., et al., *Selectively increasing inducible heat shock protein 70 via TAT-protein transduction protects neurons from nitrosative stress and excitotoxicity.* J Neurochem, 2005. **94**(2): p. 360-6.

179. Lee, J.S., J.J. Lee, and J.S. Seo, *HSP70 deficiency results in activation of c-Jun N-terminal Kinase, extracellular signal-regulated kinase, and caspase-3 in hyperosmolarity-induced apoptosis.* J Biol Chem, 2005. **280**(8): p. 6634-41.
180. Pardue, S., et al., *Elevated levels of inducible heat shock 70 proteins in human brain.* Neurobiol Aging, 2006.
181. Matsumori, Y., et al., *Hsp70 overexpression sequesters AIF and reduces neonatal hypoxic/ischemic brain injury.* J Cereb Blood Flow Metab, 2005. **25**(7): p. 899-910.
182. Chen, L. and X. Gao, *Neuronal apoptosis induced by endoplasmic reticulum stress.* Neurochem Res, 2002. **27**(9): p. 891-8.
183. Shibata, M., et al., *Activation of caspase-12 by endoplasmic reticulum stress induced by transient middle cerebral artery occlusion in mice.* Neuroscience, 2003. **118**(2): p. 491-9.
184. Benavides, A., et al., *CHOP plays a pivotal role in the astrocyte death induced by oxygen and glucose deprivation.* Glia, 2005. **52**(4): p. 261-75.
185. Jeon, G.S., et al., *Glial expression of the 90-kDa heat shock protein (HSP90) and the 94-kDa glucose-regulated protein (GRP94) following an excitotoxic lesion in the mouse hippocampus.* Glia, 2004. **48**(3): p. 250-8.
186. Yamagishi, N., et al., *Enhancement of oxidative stress-induced apoptosis by Hsp105alpha in mouse embryonal F9 cells.* Eur J Biochem, 2002. **269**(16): p. 4143-51.
187. Mosser, D.D., S. Ho, and J.R. Glover, *Saccharomyces cerevisiae Hsp104 enhances the chaperone capacity of human cells and inhibits heat stress-induced proapoptotic signaling.* Biochemistry, 2004. **43**(25): p. 8107-15.
188. Benn, S.C. and C.J. Woolf, *Adult neuron survival strategies--slamming on the brakes.* Nat Rev Neurosci, 2004. **5**(9): p. 686-700.
189. Kiang, J.G., *Inducible heat shock protein 70 kD and inducible nitric oxide synthase in hemorrhage/resuscitation-induced injury.* Cell Res, 2004. **14**(6): p. 450-9.
190. Garrido, C., et al., *Heat shock proteins: endogenous modulators of apoptotic cell death.* Biochem Biophys Res Commun, 2001. **286**(3): p. 433-42.
191. Ravagnan, L., et al., *Heat-shock protein 70 antagonizes apoptosis-inducing factor.* Nat Cell Biol, 2001. **3**(9): p. 839-43.
192. Beere, H.M., et al., *Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome.* Nat Cell Biol, 2000. **2**(8): p. 469-75.
193. Yenari, M.A., et al., *Antiapoptotic and anti-inflammatory mechanisms of heat-shock protein protection.* Ann N Y Acad Sci, 2005. **1053**: p. 74-83.
194. Chen, F., L.M. Demers, and X. Shi, *Upstream signal transduction of NF-kappaB activation.* Curr Drug Targets Inflamm Allergy, 2002. **1**(2): p. 137-49.
195. Mattson, M.P., *Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders.* Neuromolecular Med, 2003. **3**(2): p. 65-94.
196. Matute, C., et al., *Excitotoxicity in glial cells.* Eur J Pharmacol, 2002. **447**(2-3): p. 239-46.
197. Lynch, D.R. and R.P. Guttmann, *Excitotoxicity: perspectives based on N-methyl-D-aspartate receptor subtypes.* J Pharmacol Exp Ther, 2002. **300**(3): p. 717-23.
198. Mattson, M.P., *Calcium and neurodegeneration.* Aging Cell, 2007.
199. Hutchins, J.B. and S.W. Barger, *Why neurons die: cell death in the nervous system.* Anat Rec, 1998. **253**(3): p. 79-90.
200. Gozani, O., et al., *Life and death in paradise.* Nat Cell Biol, 2002. **4**(6): p. E159-62.
201. Pascual, J.M., et al., *Glutamate, glutamine, and GABA as substrates for the neuronal and glial compartments after focal cerebral ischemia in rats.* Stroke, 1998. **29**(5): p. 1048-56; discussion 1056-7.
202. Hertz, L. and H.R. Zielke, *Astrocytic control of glutamatergic activity: astrocytes as stars of the show.* Trends Neurosci, 2004. **27**(12): p. 735-43.
203. Maragakis, N.J. and J.D. Rothstein, *Mechanisms of Disease: astrocytes in neurodegenerative disease.* Nat Clin Pract Neurol, 2006. **2**(12): p. 679-89.
204. Wilhelmsson, U., et al., *Redefining the concept of reactive astrocytes as cells that remain within their unique domains upon reaction to injury.* Proc Natl Acad Sci U S A, 2006. **103**(46): p. 17513-8.
205. Pekny, M. and M. Nilsson, *Astrocyte activation and reactive gliosis.* Glia, 2005. **50**(4): p. 427-34.
206. Galea, E., et al., *Intrinsic regulation of brain inflammatory responses.* Cell Mol Neurobiol, 2003. **23**(4-5): p. 625-35.
207. Lee, S.C., D.W. Dickson, and C.F. Brosnan, *Interleukin-1, nitric oxide and reactive astrocytes.* Brain Behav Immun, 1995. **9**(4): p. 345-54.

208. Balasingam, V., et al., *Reactive astrogliosis in the neonatal mouse brain and its modulation by cytokines.* J Neurosci, 1994. **14**(2): p. 846-56.
209. Acarin, L., B. Gonzalez, and B. Castellano, *Neuronal, astroglial and microglial cytokine expression after an excitotoxic lesion in the immature rat brain.* Eur J Neurosci, 2000. **12**(10): p. 3505-20.
210. Acarin, L., et al., *Expression of inducible nitric oxide synthase and cyclooxygenase-2 after excitotoxic damage to the immature rat brain.* J Neurosci Res, 2002. **68**(6): p. 745-54.
211. Buisson, A., et al., *Transforming growth factor-beta and ischemic brain injury.* Cell Mol Neurobiol, 2003. **23**(4-5): p. 539-50.
212. Takuma, K., A. Baba, and T. Matsuda, *Astrocyte apoptosis: implications for neuroprotection.* Prog Neurobiol, 2004. **72**(2): p. 111-27.
213. Petito, C.K., et al., *Selective glial vulnerability following transient global ischemia in rat brain.* J Neuropathol Exp Neurol, 1998. **57**(3): p. 231-8.
214. Li, Y., et al., *In situ detection of DNA fragmentation after focal cerebral ischemia in mice.* Brain Res Mol Brain Res, 1995. **28**(1): p. 164-8.
215. Hollensworth, S.B., et al., *Glial cell type-specific responses to menadione-induced oxidative stress.* Free Radic Biol Med, 2000. **28**(8): p. 1161-74.
216. Walz, W., *Controversy surrounding the existence of discrete functional classes of astrocytes in adult gray matter.* Glia, 2000. **31**(2): p. 95-103.
217. Lukaszevicz, A.C., et al., *High sensitivity of protoplasmic cortical astroglia to focal ischemia.* J Cereb Blood Flow Metab, 2002. **22**(3): p. 289-98.
218. Zhang, Y., C. Goodyer, and A. LeBlanc, *Selective and protracted apoptosis in human primary neurons microinjected with active caspase-3, -6, -7, and -8.* J Neurosci, 2000. **20**(22): p. 8384-9.
219. Chan, P.H., et al., *Overexpression of SOD1 in transgenic rats protects vulnerable neurons against ischemic damage after global cerebral ischemia and reperfusion.* J Neurosci, 1998. **18**(20): p. 8292-9.
220. Kim, M.S., et al., *Protective effects of morphine in peroxynitrite-induced apoptosis of primary rat neonatal astrocytes: potential involvement of G protein and phosphatidylinositol 3-kinase (PI3 kinase).* Biochem Pharmacol, 2001. **61**(7): p. 779-86.
221. Hidalgo, J., et al., *Roles of the metallothionein family of proteins in the central nervous system.* Brain Res Bull, 2001. **55**(2): p. 133-45.
222. Sharif, A., et al., *PEA-15 modulates TNFalpha intracellular signaling in astrocytes.* Ann N Y Acad Sci, 2003. **1010**: p. 43-50.
223. Renault, F., et al., *The multifunctional protein PEA-15 is involved in the control of apoptosis and cell cycle in astrocytes.* Biochem Pharmacol, 2003. **66**(8): p. 1581-8.
224. Xu, L., et al., *Susceptibility to apoptosis varies with time in culture for murine neurons and astrocytes: changes in gene expression and activity.* Neurol Res, 2004. **26**(6): p. 632-43.
225. Mehta, S.L., N. Manhas, and R. Raghubir, *Molecular targets in cerebral ischemia for developing novel therapeutics.* Brain Res Rev, 2007. **54**(1): p. 34-66.
226. Lee, E.H., et al., *Induction of integrin-associated protein (IAP) mRNA expression during memory consolidation in rat hippocampus.* Eur J Neurosci, 2000. **12**(3): p. 1105-12.
227. Ouyang, Y.B., et al., *Overexpression of inducible heat shock protein 70 and its mutants in astrocytes is associated with maintenance of mitochondrial physiology during glucose deprivation stress.* Cell Stress Chaperones, 2006. **11**(2): p. 180-6.
228. Giffard, R. and Y. Ouyang, *Effect of overexpression of protective genes on mitochondrial function of stressed astrocytes.* J Bioenerg Biomembr, 2004. **36**(4): p. 313-5.
229. Giffard, R.G. and R.A. Swanson, *Ischemia-induced programmed cell death in astrocytes.* Glia, 2005. **50**(4): p. 299-306.
230. Beer, R., et al., *Temporal and spatial profile of caspase 8 expression and proteolysis after experimental traumatic brain injury.* J Neurochem, 2001. **78**(4): p. 862-73.
231. Newcomb, J.K., et al., *Temporal profile of apoptotic-like changes in neurons and astrocytes following controlled cortical impact injury in the rat.* Exp Neurol, 1999. **158**(1): p. 76-88.
232. Biran, V., et al., *Glial activation in white matter following ischemia in the neonatal P7 rat brain.* Exp Neurol, 2006.

233. Loos, M., M. Dihne, and F. Block, *Tumor necrosis factor-alpha expression in areas of remote degeneration following middle cerebral artery occlusion of the rat.* Neuroscience, 2003. **122**(2): p. 373-80.
234. Aoyama, K., et al., *Acidosis causes endoplasmic reticulum stress and caspase-12-mediated astrocyte death.* J Cereb Blood Flow Metab, 2005. **25**(3): p. 358-70.
235. Larner, S.F., et al., *Increased expression and processing of caspase-12 after traumatic brain injury in rats.* J Neurochem, 2004. **88**(1): p. 78-90.
236. Ientile, R., et al., *Cystamine inhibits transglutaminase and caspase-3 cleavage in glutamate-exposed astroglial cells.* J Neurosci Res, 2003. **74**(1): p. 52-9.
237. Yabe, T., et al., *Pigment epithelium-derived factor induces pro-inflammatory genes in neonatal astrocytes through activation of NF-kappa B and CREB.* Glia, 2005. **50**(3): p. 223-34.
238. DeCoster, M.A., *Group III secreted phospholipase A2 causes apoptosis in rat primary cortical neuronal cultures.* Brain Res, 2003. **988**(1-2): p. 20-8.
239. Bonini, P., et al., *Oxidative stress induces p53-mediated apoptosis in glia: p53 transcription-independent way to die.* J Neurosci Res, 2004. **75**(1): p. 83-95.
240. Ginis, I., et al., *Tumor necrosis factor and reactive oxygen species cooperative cytotoxicity is mediated via inhibition of NF-kappaB.* Mol Med, 2000. **6**(12): p. 1028-41.
241. Juknat, A.A., et al., *Melatonin prevents hydrogen peroxide-induced Bax expression in cultured rat astrocytes.* J Pineal Res, 2005. **38**(2): p. 84-92.
242. Takuma, K., et al., *Apoptosis in Ca2 + reperfusion injury of cultured astrocytes: roles of reactive oxygen species and NF-kappaB activation.* Eur J Neurosci, 1999. **11**(12): p. 4204-12.
243. Jou, M.J., et al., *Visualization of the antioxidative effects of melatonin at the mitochondrial level during oxidative stress-induced apoptosis of rat brain astrocytes.* J Pineal Res, 2004. **37**(1): p. 55-70.
244. Zhu, K., H. Lu, and W. Ying, *Post-treatment with the Ca(2+)-Mg(2+)-endonuclease inhibitor aurintricarboxylic acid prevents peroxynitrite-induced DNA damage and death of murine astrocytes.* Biochem Biophys Res Commun, 2006. **344**(3): p. 881-6.
245. Sims, N.R., M. Nilsson, and H. Muyderman, *Mitochondrial glutathione: a modulator of brain cell death.* J Bioenerg Biomembr, 2004. **36**(4): p. 329-33.
246. Almeida, A., S. Moncada, and J.P. Bolanos, *Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway.* Nat Cell Biol, 2004. **6**(1): p. 45-51.
247. Jou, M.J., et al., *Critical role of mitochondrial reactive oxygen species formation in visible laser irradiation-induced apoptosis in rat brain astrocytes (RBA-1).* J Biomed Sci, 2002. **9**(6 Pt 1): p. 507-16.
248. Lee, W.T., et al., *UV-vulnerability of human papilloma virus type-16 E7-expressing astrocytes is associated with mitochondrial membrane depolarization and caspase-3 activation.* Mol Cells, 2002. **14**(2): p. 288-94.
249. Ginis, I., et al., *TNF-alpha pretreatment prevents subsequent activation of cultured brain cells with TNF-alpha and hypoxia via ceramide.* Am J Physiol, 1999. **276**(5 Pt 1): p. C1171-83.
250. Hazell, A.S., *Astrocytes and manganese neurotoxicity.* Neurochem Int, 2002. **41**(4): p. 271-7.
251. Ouyang, Y.B. and R.G. Giffard, *Changes in astrocyte mitochondrial function with stress: effects of Bcl-2 family proteins.* Neurochem Int, 2004. **45**(2-3): p. 371-9.
252. Dugan, L.L. and J.S. Kim-Han, *Astrocyte mitochondria in in vitro models of ischemia.* J Bioenerg Biomembr, 2004. **36**(4): p. 317-21.
253. Hur, J., et al., *Induction of caspase-11 by inflammatory stimuli in rat astrocytes: lipopolysaccharide induction through p38 mitogen-activated protein kinase pathway.* FEBS Lett, 2001. **507**(2): p. 157-62.
254. Suk, K., S.Y. Kim, and H. Kim, *Essential role of caspase-11 in activation-induced cell death of rat astrocytes.* J Neurochem, 2002. **80**(2): p. 230-8.
255. Abbracchio, M.P., et al., *A novel action for adenosine: apoptosis of astroglial cells in rat brain primary cultures.* Biochem Biophys Res Commun, 1995. **213**(3): p. 908-15.
256. Hu, J. and L.J. Van Eldik, *S100 beta induces apoptotic cell death in cultured astrocytes via a nitric oxide-dependent pathway.* Biochim Biophys Acta, 1996. **1313**(3): p. 239-45.
257. Watts, L.T., et al., *Astrocytes protect neurons from ethanol-induced oxidative stress and apoptotic death.* J Neurosci Res, 2005. **80**(5): p. 655-66.

258. Pascual, M., et al., *Ceramide pathways modulate ethanol-induced cell death in astrocytes.* J Neurochem, 2003. **87**(6): p. 1535-45.
259. Minambres, R., et al., *The RhoA/ROCK-I/MLC pathway is involved in the ethanol-induced apoptosis by anoikis in astrocytes.* J Cell Sci, 2006. **119**(Pt 2): p. 271-82.
260. Enomoto, R., et al., *Involvement of histone phosphorylation in apoptosis of human astrocytes after exposure to saline solution.* Neurochem Int, 2004. **44**(6): p. 459-67.
261. Schonfeld, P., S. Kahlert, and G. Reiser, *A study of the cytotoxicity of branched-chain phytanic acid with mitochondria and rat brain astrocytes.* Exp Gerontol, 2006.
262. Benjelloun, N., et al., *Inflammatory responses in the cerebral cortex after ischemia in the P7 neonatal Rat.* Stroke, 1999. **30**(9): p. 1916-23; discussion 1923-4.
263. Benjelloun, N., et al., *Apoptotic mitochondrial pathway in neurones and astrocytes after neonatal hypoxia-ischaemia in the rat brain.* Neuropathol Appl Neurobiol, 2003. **29**(4): p. 350-60.
264. Stoll, G., S. Jander, and M. Schroeter, *Inflammation and glial responses in ischemic brain lesions.* Prog Neurobiol, 1998. **56**(2): p. 149-71.
265. Ruscher, K., et al., *Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an in vitro model.* J Neurosci, 2002. **22**(23): p. 10291-301.
266. Takuma, K., *[Delayed apoptosis and its regulation in astrocytes].* Yakugaku Zasshi, 2001. **121**(9): p. 663-9.
267. Giffard, R.G., et al., *Chaperones, protein aggregation, and brain protection from hypoxic/ischemic injury.* J Exp Biol, 2004. **207**(Pt 18): p. 3213-20.
268. Wang, Q., et al., *Dietary grape supplement ameliorates cerebral ischemia-induced neuronal death in gerbils.* Mol Nutr Food Res, 2005. **49**(5): p. 443-51.
269. Nakase, T., et al., *Increased apoptosis and inflammation after focal brain ischemia in mice lacking connexin43 in astrocytes.* Am J Pathol, 2004. **164**(6): p. 2067-75.
270. Bambrick, L., T. Kristian, and G. Fiskum, *Astrocyte mitochondrial mechanisms of ischemic brain injury and neuroprotection.* Neurochem Res, 2004. **29**(3): p. 601-8.
271. Guan, J., et al., *Neuroprotective effects of the N-terminal tripeptide of insulin-like growth factor-1, glycine-proline-glutamate (GPE) following intravenous infusion in hypoxic-ischemic adult rats.* Neuropharmacology, 2004. **47**(6): p. 892-903.
272. Rothstein, R.P. and S.W. Levison, *Gray matter oligodendrocyte progenitors and neurons die caspase-3 mediated deaths subsequent to mild perinatal hypoxic/ischemic insults.* Dev Neurosci, 2005. **27**(2-4): p. 149-59.
273. Khan, M., et al., *S-Nitrosoglutathione reduces inflammation and protects brain against focal cerebral ischemia in a rat model of experimental stroke.* J Cereb Blood Flow Metab, 2005. **25**(2): p. 177-92.
274. Nakase, T., S. Fushiki, and C.C. Naus, *Astrocytic gap junctions composed of connexin 43 reduce apoptotic neuronal damage in cerebral ischemia.* Stroke, 2003. **34**(8): p. 1987-93.
275. Nakase, T., et al., *Neuroprotective role of astrocytic gap junctions in ischemic stroke.* Cell Commun Adhes, 2003. **10**(4-6): p. 413-7.
276. Johnston, M.V., *Excitotoxicity in perinatal brain injury.* Brain Pathol, 2005. **15**(3): p. 234-40.
277. Franke, H., et al., *P2X7 receptor expression after ischemia in the cerebral cortex of rats.* J Neuropathol Exp Neurol, 2004. **63**(7): p. 686-99.
278. Jantzie, L.L., P.Y. Cheung, and K.G. Todd, *Doxycycline reduces cleaved caspase-3 and microglial activation in an animal model of neonatal hypoxia-ischemia.* J Cereb Blood Flow Metab, 2005. **25**(3): p. 314-24.
279. Wang, Q., et al., *Neuroprotective mechanisms of curcumin against cerebral ischemia-induced neuronal apoptosis and behavioral deficits.* J Neurosci Res, 2005. **82**(1): p. 138-48.
280. Xia, C.F., et al., *Adrenomedullin gene delivery protects against cerebral ischemic injury by promoting astrocyte migration and survival.* Hum Gene Ther, 2004. **15**(12): p. 1243-54.
281. Sugawara, T., et al., *Mitochondrial release of cytochrome c corresponds to the selective vulnerability of hippocampal CA1 neurons in rats after transient global cerebral ischemia.* J Neurosci, 1999. **19**(22): p. RC39.
282. Wiggins, A.K., P.J. Shen, and A.L. Gundlach, *Delayed, but prolonged increases in astrocytic clusterin (ApoJ) mRNA expression following acute cortical spreading depression in the rat: evidence for a role of clusterin in ischemic tolerance.* Brain Res Mol Brain Res, 2003. **114**(1): p. 20-30.

283. Aggoun-Zouaoui, D., et al., *Ultrastructural morphology of neuronal death following reversible focal ischemia in the rat.* Apoptosis, 1998. **3**(2): p. 133-41.
284. Ferrer, I., et al., *Differential c-Fos and caspase expression following kainic acid excitotoxicity.* Acta Neuropathol (Berl), 2000. **99**(3): p. 245-56.
285. Kalehua, A.N., et al., *Monocyte chemoattractant protein-1 and macrophage inflammatory protein-2 are involved in both excitotoxin-induced neurodegeneration and regeneration.* Exp Cell Res, 2004. **297**(1): p. 197-211.
286. Mothe, A.J. and C.H. Tator, *Proliferation, migration, and differentiation of endogenous ependymal region stem/progenitor cells following minimal spinal cord injury in the adult rat.* Neuroscience, 2005. **131**(1): p. 177-87.
287. Kwak, E.K., et al., *The role of inducible nitric oxide synthase following spinal cord injury in rat.* J Korean Med Sci, 2005. **20**(4): p. 663-9.
288. Bannerman, P., et al., *Astrogliosis in EAE spinal cord: derivation from radial glia, and relationships to oligodendroglia.* Glia, 2007. **55**(1): p. 57-64.
289. Knoblach, S.M., et al., *Selective caspase activation may contribute to neurological dysfunction after experimental spinal cord trauma.* J Neurosci Res, 2005. **80**(3): p. 369-80.
290. Larner, S.F., et al., *Caspase 7: increased expression and activation after traumatic brain injury in rats.* J Neurochem, 2005. **94**(1): p. 97-108.
291. Di Giovanni, S., et al., *Cell cycle inhibition provides neuroprotection and reduces glial proliferation and scar formation after traumatic brain injury.* Proc Natl Acad Sci U S A, 2005. **102**(23): p. 8333-8.
292. Penkowa, M., et al., *Astrocyte-targeted expression of IL-6 protects the CNS against a focal brain injury.* Exp Neurol, 2003. **181**(2): p. 130-48.
293. Penkowa, M., et al., *Metallothionein-I overexpression alters brain inflammation and stimulates brain repair in transgenic mice with astrocyte-targeted interleukin-6 expression.* Glia, 2003. **42**(3): p. 287-306.
294. Penkowa, M., et al., *Metallothionein prevents neurodegeneration and central nervous system cell death after treatment with gliotoxin 6-aminonicotinamide.* J Neurosci Res, 2004. **77**(1): p. 35-53.
295. Clausen, F., et al., *Oxygen free radical-dependent activation of extracellular signal-regulated kinase mediates apoptosis-like cell death after traumatic brain injury.* J Neurotrauma, 2004. **21**(9): p. 1168-82.
296. Beer, R., et al., *Temporal profile and cell subtype distribution of activated caspase-3 following experimental traumatic brain injury.* J Neurochem, 2000. **75**(3): p. 1264-73.
297. Barbeito, L.H., et al., *A role for astrocytes in motor neuron loss in amyotrophic lateral sclerosis.* Brain Res Brain Res Rev, 2004. **47**(1-3): p. 263-74.
298. Consilvio, C., A.M. Vincent, and E.L. Feldman, *Neuroinflammation, COX-2, and ALS--a dual role?* Exp Neurol, 2004. **187**(1): p. 1-10.
299. Vargas, M.R., et al., *Fibroblast growth factor-1 induces heme oxygenase-1 via nuclear factor erythroid 2-related factor 2 (Nrf2) in spinal cord astrocytes: consequences for motor neuron survival.* J Biol Chem, 2005. **280**(27): p. 25571-9.
300. Lechuga-Sancho, A.M., et al., *Activation of the intrinsic cell death pathway, increased apoptosis and modulation of astrocytes in the cerebellum of diabetic rats.* Neurobiol Dis, 2006. **23**(2): p. 290-9.
301. Feit-Leichman, R.A., et al., *Vascular damage in a mouse model of diabetic retinopathy: relation to neuronal and glial changes.* Invest Ophthalmol Vis Sci, 2005. **46**(11): p. 4281-7.
302. Smale, G., et al., *Evidence for apoptotic cell death in Alzheimer's disease.* Exp Neurol, 1995. **133**(2): p. 225-30.
303. Su, J.H., et al., *DNA damage and activated caspase-3 expression in neurons and astrocytes: evidence for apoptosis in frontotemporal dementia.* Exp Neurol, 2000. **163**(1): p. 9-19.
304. Mattson, M.P. and S.L. Chan, *Neuronal and glial calcium signaling in Alzheimer's disease.* Cell Calcium, 2003. **34**(4-5): p. 385-97.
305. Kobayashi, K., et al., *Apoptosis of astrocytes with enhanced lysosomal activity and oligodendrocytes in white matter lesions in Alzheimer's disease.* Neuropathol Appl Neurobiol, 2002. **28**(3): p. 238-51.
306. Broe, M., J. Kril, and G.M. Halliday, *Astrocytic degeneration relates to the severity of disease in frontotemporal dementia.* Brain, 2004. **127**(Pt 10): p. 2214-20.
307. Emerit, J., M. Edeas, and F. Bricaire, *Neurodegenerative diseases and oxidative stress.* Biomed Pharmacother, 2004. **58**(1): p. 39-46.

308. Garden, G.A., et al., *Caspase cascades in human immunodeficiency virus-associated neurodegeneration.* J Neurosci, 2002. **22**(10): p. 4015-24.
309. Guillemin, G.J., L. Wang, and B.J. Brew, *Quinolinic acid selectively induces apoptosis of human astrocytes: potential role in AIDS dementia complex.* J Neuroinflammation, 2005. **2**: p. 16.
310. Jellinger, K.A., *Cell death mechanisms in Parkinson's disease.* J Neural Transm, 2000. **107**(1): p. 1-29.
311. Jellinger, K.A. and C. Stadelmann, *Mechanisms of cell death in neurodegenerative disorders.* J Neural Transm Suppl, 2000. **59**: p. 95-114.
312. Kobayashi, K., et al., *Correlation between astrocyte apoptosis and Alzheimer changes in gray matter lesions in Alzheimer's disease.* J Alzheimers Dis, 2004. **6**(6): p. 623-32; discussion 673-81.
313. Saas, P., et al., *CD95 (Fas/Apo-1) as a receptor governing astrocyte apoptotic or inflammatory responses: a key role in brain inflammation?* J Immunol, 1999. **162**(4): p. 2326-33.
314. Ueberham, U., A. Hessel, and T. Arendt, *Cyclin C expression is involved in the pathogenesis of Alzheimer's disease.* Neurobiol Aging, 2003. **24**(3): p. 427-35.
315. Wu, Y.P., et al., *Apoptosis accompanied by up-regulation of TNF-alpha death pathway genes in the brain of Niemann-Pick type C disease.* Mol Genet Metab, 2005. **84**(1): p. 9-17.
316. Vannucci, S.J. and H. Hagberg, *Hypoxia-ischemia in the immature brain.* J Exp Biol, 2004. **207**(Pt 18): p. 3149-54.
317. Oppenheim, R.W., et al., *Naturally occurring and induced neuronal death in the chick embryo in vivo requires protein and RNA synthesis: evidence for the role of cell death genes.* Dev Biol, 1990. **138**(1): p. 104-13.
318. Vexler, Z.S. and D.M. Ferriero, *Molecular and biochemical mechanisms of perinatal brain injury.* Semin Neonatol, 2001. **6**(2): p. 99-108.
319. Blomgren, K. and H. Hagberg, *Free radicals, mitochondria, and hypoxia-ischemia in the developing brain.* Free Radic Biol Med, 2006. **40**(3): p. 388-97.
320. Takai, H., et al., *NMDA-induced apoptosis in the developing rat brain.* Exp Toxicol Pathol, 2003. **55**(1): p. 33-7.
321. McQuillen, P.S. and D.M. Ferriero, *Selective vulnerability in the developing central nervous system.* Pediatr Neurol, 2004. **30**(4): p. 227-35.
322. Acarin, L., et al., *Astroglial nitration after postnatal excitotoxic damage: correlation with nitric oxide sources, cytoskeletal, apoptotic and antioxidant proteins.* J Neurotrauma, 2005. **22**(1): p. 189-200.
323. Acarin, L., et al., *Quantitative analysis of microglial reaction to a cortical excitotoxic lesion in the early postnatal brain.* Exp Neurol, 1997. **147**(2): p. 410-7.
324. Peluffo, H., et al., *Cu/Zn superoxide dismutase expression in the postnatal rat brain following an excitotoxic injury.* J Neuroinflammation, 2005. **2**(1): p. 12.
325. Faiz, M., et al., *Antioxidant Cu/Zn SOD: Expression in postnatal brain progenitor cells.* Neurosci Lett, 2006.
326. Acarin, L., B. Gonzalez, and B. Castellano, *Glial activation in the immature rat brain: implication of inflammatory transcription factors and cytokine expression.* Prog Brain Res, 2001. **132**: p. 375-89.
327. Acarin, L., B. Gonzalez, and B. Castellano, *Stat3 and NFkappaB glial expression after excitotoxic damage to the postnatal brain.* Neuroreport, 1998. **9**(12): p. 2869-73.
328. Acarin, L., et al., *Primary cortical glial reaction versus secondary thalamic glial response in the excitotoxically injured young brain: astroglial response and metallothionein expression.* Neuroscience, 1999. **92**(3): p. 827-39.
329. Martin, L.J., et al., *Hypoxia-ischemia causes abnormalities in glutamate transporters and death of astroglia and neurons in newborn striatum.* Ann Neurol, 1997. **42**(3): p. 335-48.
330. Micoud, F., B. Mandrand, and C. Malcus-Vocanson, *Comparison of several techniques for the detection of apoptotic astrocytes in vitro.* Cell Prolif, 2001. **34**(2): p. 99-113.
331. Pulera, M.R., et al., *Apoptosis in a neonatal rat model of cerebral hypoxia-ischemia.* Stroke, 1998. **29**(12): p. 2622-30.
332. Charriaut-Marlangue, C., et al., *Apoptosis and necrosis after reversible focal ischemia: an in situ DNA fragmentation analysis.* J Cereb Blood Flow Metab, 1996. **16**(2): p. 186-94.
333. Nakajima, W., et al., *Apoptosis has a prolonged role in the neurodegeneration after hypoxic ischemia in the newborn rat.* J Neurosci, 2000. **20**(21): p. 7994-8004.
334. Didenko, V.V., et al., *Caspase-3-dependent and -independent apoptosis in focal brain ischemia.* Mol Med, 2002. **8**(7): p. 347-52.

335. Johnston, M.V., W. Nakajima, and H. Hagberg, *Mechanisms of hypoxic neurodegeneration in the developing brain.* Neuroscientist, 2002. **8**(3): p. 212-20.
336. Auten, R.L., M.H. Whorton, and S. Nicholas Mason, *Blocking neutrophil influx reduces DNA damage in hyperoxia-exposed newborn rat lung.* Am J Respir Cell Mol Biol, 2002. **26**(4): p. 391-7.
337. Puka-Sundvall, M., et al., *Subcellular distribution of calcium and ultrastructural changes after cerebral hypoxia-ischemia in immature rats.* Brain Res Dev Brain Res, 2000. **125**(1-2): p. 31-41.
338. Northington, F.J., E.M. Graham, and L.J. Martin, *Apoptosis in perinatal hypoxic-ischemic brain injury: how important is it and should it be inhibited?* Brain Res Brain Res Rev, 2005. **50**(2): p. 244-57.
339. Zhu, C., et al., *Intraischemic mild hypothermia prevents neuronal cell death and tissue loss after neonatal cerebral hypoxia-ischemia.* Eur J Neurosci, 2006. **23**(2): p. 387-93.
340. Northington, F.J., et al., *Delayed neurodegeneration in neonatal rat thalamus after hypoxia-ischemia is apoptosis.* J Neurosci, 2001. **21**(6): p. 1931-8.
341. Chan, W.Y., et al., *Proliferation and apoptosis in the developing human neocortex.* Anat Rec, 2002. **267**(4): p. 261-76.
342. McQuillen, P.S., et al., *Selective vulnerability of subplate neurons after early neonatal hypoxia-ischemia.* J Neurosci, 2003. **23**(8): p. 3308-15.
343. Ferrer, I., et al., *Caspase-dependent and caspase-independent signalling of apoptosis in the penumbra following middle cerebral artery occlusion in the adult rat.* Neuropathol Appl Neurobiol, 2003. **29**(5): p. 472-81.
344. Zhu, C., et al., *Involvement of apoptosis-inducing factor in neuronal death after hypoxia-ischemia in the neonatal rat brain.* J Neurochem, 2003. **86**(2): p. 306-17.
345. Susin, S.A., et al., *Molecular characterization of mitochondrial apoptosis-inducing factor.* Nature, 1999. **397**(6718): p. 441-6.
346. Wang, H., et al., *Apoptosis-inducing factor substitutes for caspase executioners in NMDA-triggered excitotoxic neuronal death.* J Neurosci, 2004. **24**(48): p. 10963-73.
347. Chua, C.C., et al., *Overexpression of IAP-2 attenuates apoptosis and protects against myocardial ischemia/reperfusion injury in transgenic mice.* Biochim Biophys Acta, 2007.
348. Goffredo, D., et al., *Prevention of cytosolic IAPs degradation: a potential pharmacological target in Huntington's Disease.* Pharmacol Res, 2005. **52**(2): p. 140-50.
349. Kam, P.C. and N.I. Ferch, *Apoptosis: mechanisms and clinical implications.* Anaesthesia, 2000. **55**(11): p. 1081-93.
350. Holler, N., et al., *Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule.* Nat Immunol, 2000. **1**(6): p. 489-95.
351. Pellegrini, M., et al., *FADD and caspase-8 are required for cytokine-induced proliferation of hemopoietic progenitor cells.* Blood, 2005. **106**(5): p. 1581-9.
352. Su, H., et al., *Requirement for caspase-8 in NF-kappaB activation by antigen receptor.* Science, 2005. **307**(5714): p. 1465-8.
353. Matute, C., et al., *Excitotoxic damage to white matter.* J Anat, 2007. **210**(6): p. 693-702.
354. Matute, C., *Oligodendrocyte NMDA receptors: a novel therapeutic target.* Trends Mol Med, 2006. **12**(7): p. 289-92.
355. Tekkok, S.B., B.T. Faddis, and M.P. Goldberg, *AMPA/kainate receptors mediate axonal morphological disruption in hypoxic white matter.* Neurosci Lett, 2005. **382**(3): p. 275-9.
356. Folkerth, R.D., *Periventricular leukomalacia: overview and recent findings.* Pediatr Dev Pathol, 2006. **9**(1): p. 3-13.
357. Olivier, P., et al., *Prenatal ischemia and white matter damage in rats.* J Neuropathol Exp Neurol, 2005. **64**(11): p. 998-1006.
358. Shibata, M., et al., *Caspases determine the vulnerability of oligodendrocytes in the ischemic brain.* J Clin Invest, 2000. **106**(5): p. 643-53.
359. Casaccia-Bonnefil, P. and A. Liu, *Relationship between cell cycle molecules and onset of oligodendrocyte differentiation.* J Neurosci Res, 2003. **72**(1): p. 1-11.
360. Hisahara, S., H. Okano, and M. Miura, *Caspase-mediated oligodendrocyte cell death in the pathogenesis of autoimmune demyelination.* Neurosci Res, 2003. **46**(4): p. 387-97.
361. Griffiths, I.R., et al., *Transgenic and natural mouse models of proteolipid protein (PLP)-related dysmyelination and demyelination.* Brain Pathol, 1995. **5**(3): p. 275-81.

362. Racke, M.M., et al., *Activation of caspase-3 alone is insufficient for apoptotic morphological changes in human neuroblastoma cells.* J Neurochem, 2002. **80**(6): p. 1039-48.
363. Nakagawa, T., et al., *Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta.* Nature, 2000. **403**(6765): p. 98-103.
364. Lee, W., et al., *ER stress-induced caspase-12 activation is inhibited by PKC in neuronal cells.* Apoptosis, 2005. **10**(2): p. 407-15.
365. Garden, G.A. and R.S. Morrison, *The multiple roles of p53 in the pathogenesis of HIV associated dementia.* Biochem Biophys Res Commun, 2005. **331**(3): p. 799-809.
366. Martin, L.J., et al., *Injury-induced apoptosis of neurons in adult brain is mediated by p53-dependent and p53-independent pathways and requires Bax.* J Comp Neurol, 2001. **433**(3): p. 299-311.
367. Cummings, B.S. and R.G. Schnellmann, *Cisplatin-induced renal cell apoptosis: caspase 3-dependent and -independent pathways.* J Pharmacol Exp Ther, 2002. **302**(1): p. 8-17.
368. Suzuki, Y., Y. Nakabayashi, and R. Takahashi, *Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death.* Proc Natl Acad Sci U S A, 2001. **98**(15): p. 8662-7.
369. Eckelman, B.P., G.S. Salvesen, and F.L. Scott, *Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family.* EMBO Rep, 2006. **7**(10): p. 988-94.
370. Lee, J.S., et al., *Heat shock protein 27 interacts with vimentin and prevents insolubilization of vimentin subunits induced by cadmium.* Exp Mol Med, 2005. **37**(5): p. 427-35.
371. Bruey, J.M., et al., *Hsp27 negatively regulates cell death by interacting with cytochrome c.* Nat Cell Biol, 2000. **2**(9): p. 645-52.
372. Perng, M.D., et al., *Intermediate filament interactions can be altered by HSP27 and alphaB-crystallin.* J Cell Sci, 1999. **112 (Pt 13)**: p. 2099-112.
373. Bossenmeyer-Pourie, C., V. Koziel, and J.L. Daval, *CPP32/CASPASE-3-like proteases in hypoxia-induced apoptosis in developing brain neurons.* Brain Res Mol Brain Res, 1999. **71**(2): p. 225-37.
374. Sharif, A., et al., *The expression of PEA-15 (phosphoprotein enriched in astrocytes of 15 kDa) defines subpopulations of astrocytes and neurons throughout the adult mouse brain.* Neuroscience, 2004. **126**(2): p. 263-75.
375. Renault-Mihara, F., et al., *Phosphoprotein enriched in astrocytes-15 kDa expression inhibits astrocyte migration by a protein kinase C delta-dependent mechanism.* Mol Biol Cell, 2006. **17**(12): p. 5141-52.
376. Murphy, S., et al., *Synthesis of nitric oxide in CNS glial cells.* Trends Neurosci, 1993. **16**(8): p. 323-8.
377. Benhar, M., M.T. Forrester, and J.S. Stamler, *Nitrosative stress in the ER: a new role for S-nitrosylation in neurodegenerative diseases.* ACS Chem Biol, 2006. **1**(6): p. 355-8.
378. Benhar, M. and J.S. Stamler, *A central role for S-nitrosylation in apoptosis.* Nat Cell Biol, 2005. **7**(7): p. 645-6.
379. Rossig, L., et al., *Nitric oxide inhibits caspase-3 by S-nitrosation in vivo.* J Biol Chem, 1999. **274**(11): p. 6823-6.
380. Hoffmann, J., et al., *Aging enhances the sensitivity of endothelial cells toward apoptotic stimuli: important role of nitric oxide.* Circ Res, 2001. **89**(8): p. 709-15.
381. Oomman, S., et al., *Active caspase-3 expression during postnatal development of rat cerebellum is not systematically or consistently associated with apoptosis.* J Comp Neurol, 2004. **476**(2): p. 154-73.
382. Hetz, C.A., V. Torres, and A.F. Quest, *Beyond apoptosis: nonapoptotic cell death in physiology and disease.* Biochem Cell Biol, 2005. **83**(5): p. 579-88.
383. Alam, A., et al., *Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells.* J Exp Med, 1999. **190**(12): p. 1879-90.
384. Fernando, P., et al., *Caspase 3 activity is required for skeletal muscle differentiation.* Proc Natl Acad Sci U S A, 2002. **99**(17): p. 11025-30.
385. Fernando, P., S. Brunette, and L.A. Megeney, *Neural stem cell differentiation is dependent upon endogenous caspase 3 activity.* Faseb J, 2005. **19**(12): p. 1671-3.
386. Fernando, P. and L.A. Megeney, *Is caspase-dependent apoptosis only cell differentiation taken to the extreme?* Faseb J, 2007. **21**(1): p. 8-17.
387. Ramuz, O., et al., *Constitutive nuclear localization and initial cytoplasmic apoptotic activation of endogenous caspase-3 evidenced by confocal microscopy.* Int J Exp Pathol, 2003. **84**(2): p. 75-81.

388. Fuentes-Prior, P. and G.S. Salvesen, *The protein structures that shape caspase activity, specificity, activation and inhibition.* Biochem J, 2004. **384**(Pt 2): p. 201-32.
389. Noyan-Ashraf, M.H., F. Brandizzi, and B.H. Juurlink, *Constitutive nuclear localization of activated caspase 3 in subpopulations of the astroglial family of cells.* Glia, 2005. **49**(4): p. 588-93.
390. Brockstedt, E., et al., *Identification of apoptosis-associated proteins in a human Burkitt lymphoma cell line. Cleavage of heterogeneous nuclear ribonucleoprotein A1 by caspase 3.* J Biol Chem, 1998. **273**(43): p. 28057-64.
391. Rao, V.L., et al., *Antisense knockdown of the glial glutamate transporter GLT-1 exacerbates hippocampal neuronal damage following traumatic injury to rat brain.* Eur J Neurosci, 2001. **13**(1): p. 119-28.

8. APPENDIX

8.1. PUBLICATIONS

Sonia Villapol has elaborated the following articles as a Ph.D. student:

1. Faiz M, Acarin L, Peluffo H, **Villapol S**, Castellano B, Gonzalez B. *Antioxidant Cu/Zn SOD:expression in postnatal brain progenitor cells.* Neurosci Lett. Jun19;401(1-2):71-6, (2006).
2. Acarin L.,**Villapol S.,** Faiz M., Rohn T.T., Castellano B. and Gonzalez B. *Caspase-3 activation in astrocytes following postnatal excitotoxic damage correlates with cytoskeletal remodeling but not with cell death or proliferation.* GLIA 55:954 - 965, (2007).
3. Spiegler M., **Villapol S.,** Biran V., Goyenvalle C., Mariani J., Renolleau S., and Charriaut-Marlangue, C. *Bilateral Changes after neonatal ischemia in the P7 rat brain.* J Neuropathol Exp Neurol. Jun;66(6):481-490, (2007).
4. **Villapol S**., Acarin L., Faiz M., Castellano B. and Gonzalez B. *Distinct spatial and temporal activation profile of apoptotic pathways in neurons and glial cells after excitotoxic damage in the neonatal rat brain.* J. Neuroscience Research. *In press,* (2007).
5. **Villapol S**., Acarin L., Faiz M., Castellano B. and Gonzalez B. *Survivin and Heat Shock Protein 25/27 colocalize with cleaved caspase-3 in surviving reactive astrocytes following excitotoxicity to the immature brain. Submitted,* (2007).
6. Faiz M, Acarin L, **Villapol S**, Shultz S., Castellano B, Gonzalez B. *Mass migration of SVZ cells to the cortex results in the generation of new neurons in the excitotoxically damaged immature rat brain. Submitted,* (2007).
7. **Villapol S.,** et al. *Astrocytic demise and survival after neonatal stroke. Manuscript,* (2007).
8. **Villapol S**., et al. *Lithium enhances proliferation and astrogliosis in the rat brain of after a traumatic injury. Manuscript,* (2007).

8.2. CONFERENCES

Thesis Work presented in scientific conferences.

1. Acarin L., **Villapol S.**, Faiz M., Rohn T.T., Castellano B. and González B. *Caspase-3 activation in astrocytes following postnatal excitotoxic damage correlates with cytoskeletal remodeling but not with cell death or proliferation.* Glial Cells in Health and Disease, 8th Glial Meeting. London, September 2007. *Poster presentation/Abstract.*
2. **Villapol S.**, *Apoptotic and antiapoptotic mechanisms in glial cells and neurons after postnatal brain damage.* II Jornades Científiques, Institut de Neurociències. UAB, Barcelona, June 2007. *Oral presentation/Abstract.*
3. Faiz, M., Acarin, L., **Villapol, S.**, Castellano, B., Gonzalez, B. *SVZ progenitor migration to areas of neurodegeneration after an excitotoxic lesion to the postnatal rat brain.* SFN, Atlanta, October 2006. *Poster presentation/Abstract..*
4. **Villapol S**. Acarin L., Faiz M., Castellano B. and Gonzalez B. *Expression of activated caspase-8 and caspase-9 following an excitotoxic injury to the immature rat brain.* FENS FORUM 2006, Vienna, Austria, July 2006. *Poster presentation/Abstract.*
5. **Villapol S.**, *Increment de l'activació de caspases després dun dany excitotòxic en el cervell immadur de rata.* Sociedat Catalana de Biología. V Simposi de Neurobiologia Experimental, December, Barcelona, 2005. *Oral presentation/Abstract.*
6. **Villapol S.**, *Muerte y supervivencia tras la activación de caspasas en el cerebro inmaduro*, Acto conmemorativo del centenario del nacimiento de Severo Ochoa. FECYT y Universidad Complutense de Madrid, 15 November, Madrid, 2005. *Poster presentation/Abstract.*
7. **Villapol S**. Acarin L., Faiz M., Castellano B. and Gonzalez B. *Expression activated caspase-8 following an excitotoxic injury to the immature rat brain.* 13th Euroconference on Apoptosis, ECDO, Budapest, Hungry, October 2005. *Poster presentation/Abstract.*

Other work presented in scientific conferences.

8. **Villapol S.**, Castellano B. and Gonzalez B. *Lithium effects in the rat brain after a cortical lesion by aspiration*. 12th Euroconference on Apoptosis, ECDO, 17-20 Chania, Greece, September 2004. Poster presentation/Abstract. *Poster presentation/Abstract.*
9. Garcia A, **Villapol S**, Alvarez CV, Smith RG, Dieguez C. *Regulation of Pit-1 expression by ghrelin and GHRP-6 through the GH secretagogue receptor*. International Congress of cell signaling and cancer, Heiderberg, Germany, April 2002. Poster presentation/Abstract. *Poster presentation/Abstract.*
10. **Villapol S.**, García A., Cañibano C., Llovo R., Smith R., Diéguez C., Álvarez C. *Ghrelin phosphorylates CREB to induce pit-1 transcription*. 10th Meeting of the European Neuroendocrine Association, ENEA, Munich, Germany, September 2002. Poster presentation/Abstract. *Poster presentation/Abstract.*
11. **Villapol S.**, García A., Cañibano C., Llovo R., Smith R., Diéguez C., Álvarez C. *A Ghrelina activa e fosforial CREB e ERK*. XV Congreso da Sociedade Galega de Endocrinoloxía, Nutrición e Metabolismo. Ferrol, Noviembre, 2002. *Poster presentation/ Abstract.*
12. **Villapol S.**, García A., Cañibano C., Llovo R., Smith R., Diéguez C., Álvarez C. *Ghrelin y Pit-1 como reguladores adenohiposisarios*. 24ª Congreso de la Sociedad Española de Endocrinología Pediátrica. SEEP, Santiago de Compostela, Mayo 2002. *Poster presentation/ Abstract.*
13. **Villapol S.**, García A., Cañibano C., Llovo R., Smith R., Diéguez C., Álvarez C. *Regulación da expresión do Factor de Transcripción adenohipofisario Pit-1 por Ghrelin.* XIV Congreso da Sociedade Galega de Endocrinoloxía, Nutrición e Metabolismo. Ourense, Noviembre 2001. *Oral presentation/Abstract.*
14. **Villapol S.,** *Cara a imnortalidade das Células e a Eliminación do Cáncer; A Telomerasa*, 3ª Xuntanza de Xoves Investigadores. Edita Xunta de Galicia, A Coruña 1998. *Oral presentation/Abstract.*
15. **Villapol S.,** *Los radicales libres y el envejecimiento*, XIV Encuentro de Jóvenes Investigadores. Edita INICE, Salamanca 1998. *Oral presentation/Abstract.*