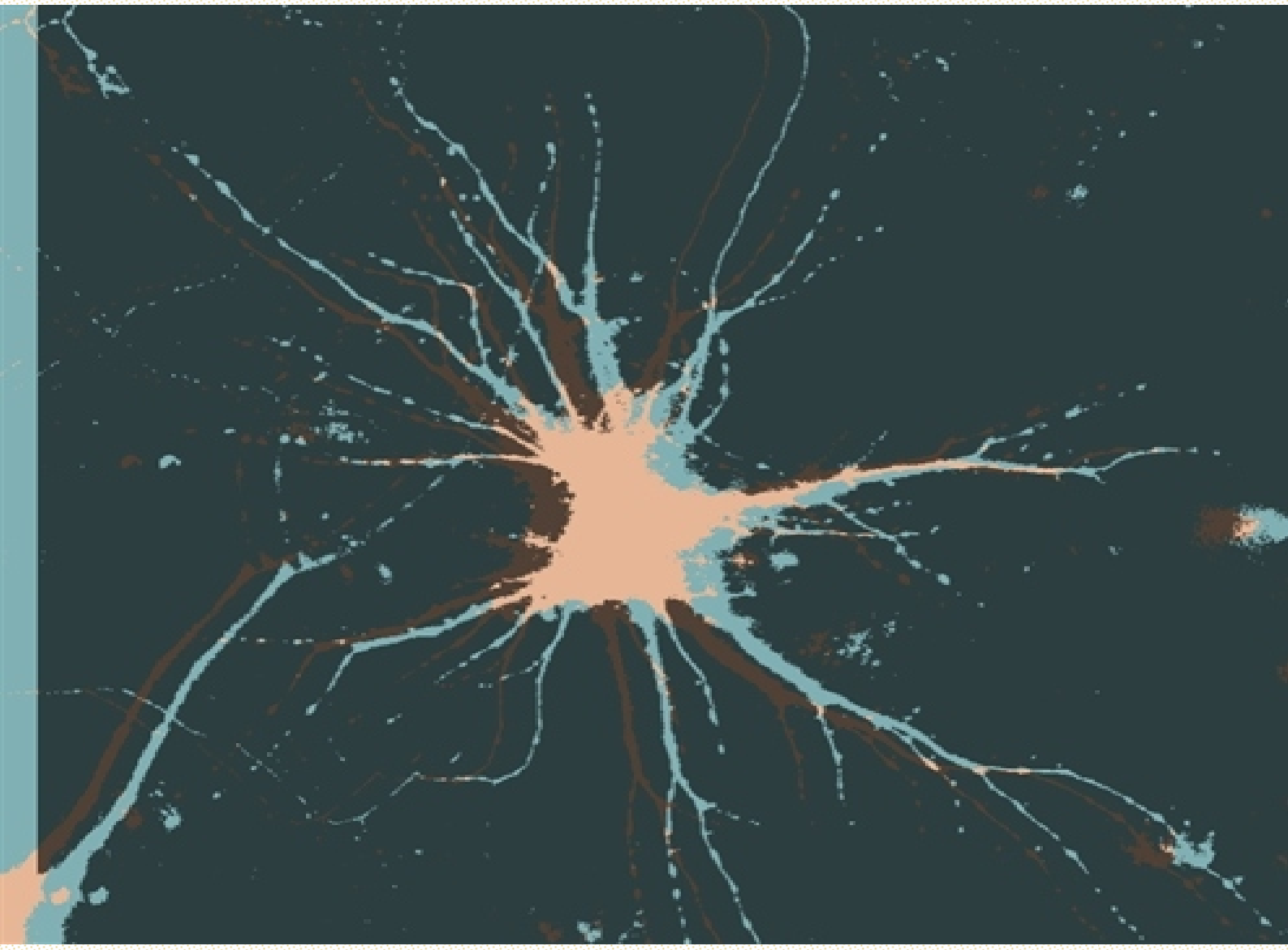


Marta Isabel Dias da Mota Vieira

Molecular Mechanisms Underlying *in vitro* Cerebral Ischemia: Multiple Neuronal Death Pathways

Tese de Doutoramento em Ciências e Tecnologias da Saúde,
orientada por Ana Luísa Carvalho e Armanda Emanuela Castro e Santos
e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro/2013

· U C ·

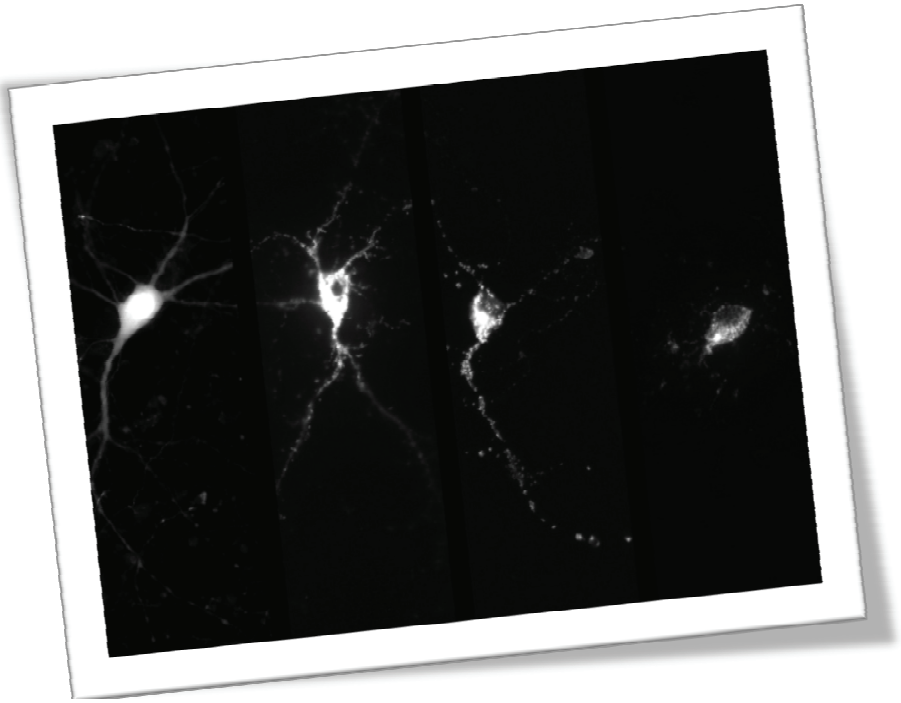
UNIVERSIDADE DE COIMBRA

Molecular Mechanisms Underlying *in vitro* Cerebral Ischemia: Multiple Neuronal Death Pathways

Mecanismos Moleculares Subjacentes à Isquemia Cerebral *in vitro*: Múltiplas Vias de Morte Neuronal

Marta Isabel Dias da Mota Vieira

2013



Dissertação apresentada à Faculdade de Farmácia da Universidade de Coimbra para prestação de provas de Doutoramento em Ciências e Tecnologias da Saúde, especialidade de Biologia Celular e Molecular.

Agradeço ao Centro de Neurociências e Biologia Celular e à Universidade de Coimbra a oportunidade de trabalhar no seu espaço, e à Fundação para a Ciência e Tecnologia que financiou o meu trabalho (bolsa de douoramento – SHFR/BD/47739/2008). Este trabalho foi financiado pela FEDER via Programa Operacional Factores de Competitividade – COMPETE e pela FCT no âmbito dos seguintes projectos: PTDC/SAU-NEU/099440/2008, PTDC/NEU-NMC/0750/2012, PTDC/SAU-NMC/12144/2010, PTDC/NEU-SCC/1351/2012 e PEst-C/SAU/LA0001/2013-2014. Os ensaios *in vivo* foram financiados pela Junta de Castilla y León (Ref. LE184A12-2).

Agradecimentos

Deste doutoramento, como da vida, o melhor que levo comigo são as pessoas e as suas lições. Como alguém sábio uma vez disse “my friends have always been the best of me”, e por isso gostaria de agradecer às pessoas mais importantes da minha vida e àquelas que de alguma forma contribuíram para o meu crescimento pessoal durante estes, por vezes longos, por vezes demasiado curtos, quatro anos de trabalho.

Gostaria de começar por agradecer a uma pessoa sem a qual esta tese não teria sido possível e sem a qual esta aventura não teria sido a mesma. A minha companheira de armas de doutoramento, Joana Fernandes. Gostaria de te agradecer, naturalmente, pela ajuda no laboratório nomeadamente pela cedência das famosas listas dos microarrays, sem as quais não teria direccionado o trabalho no sentido deste formato final. Muito obrigada por isso, mas mais importante do que tudo, muito obrigada pela tua amizade. Obrigada por estares sempre do meu lado, sempre disposta a ouvir-me. Obrigada por tornares tua missão pessoal fazer-me rir! Sem isso este doutoramento não teria tido, apesar de todos os momentos de desilusão e lágrimas, todos os momentos de diversão e alegria que teve. Não há ninguém com quem teria gostado tanto de partilhar este doutoramento como tu.

Gostaria também de agradecer às minhas orientadoras, Professora Ana Luísa Carvalho e Doutora Armanda Santos por me terem aceitado como aluna de doutoramento. Obrigada pela ajuda e orientação. Obrigada pelos ensinamentos, os quais estarão sempre presentes no meu trabalho e na minha forma de encarar a ciência. À professora Ana Luísa gostaria de agradecer o entusiasmo contagiante, que foi muito precioso para mim durante estes quatro anos. A sua visão optimista da ciência é algo que levarei sempre comigo. Obrigada pela confiança em mim depositada. Um obrigado muito especial ao Professor Carlos Duarte, por todos os ensinamentos e ajuda. Foi um prazer trabalhar consigo.

À Joana Ferreira gostaria de fazer um agradecimento muito especial. Nestes já largos anos de amizade, a tua presença foi sempre e continuará sempre a ser um porto de abrigo para mim. Obrigada por toda a ajuda e apoio incondicional, no laboratório e fora dele.

À Aninhas e à Ritinha gostaria de dizer que a vossa amizade é um dos bens mais preciosos que levo comigo deste laboratório. Obrigada por todos os momentos de carinho e amizade, e também pelos momentos de palhaçada que muito me ajudam a ter energia para me levantar todas as manhãs para continuar a fazer o meu caminho. As memórias de todos os momentos por que passei com estas quatro grandes amigas, Ritinha, Aninhas, Joaninha e Fernandinhas hão-de acompanhar-me sempre. A vossa amizade faz de mim uma pessoa melhor. Enquanto investigadora olho para vocês com admiração e espero um dia poder chegar ao vosso nível.

Às “meninas” Joana, Dani, Lena e Paulo gostaria de agradecer a incondicional amizade. Mesmo que estejamos cada um num canto diferente do mundo, a amizade que nos une é algo que nunca me abandona, vocês estão sempre comigo, onde quer que eu esteja. Estes quatro anos foram marcados por muitas mudanças nas vidas de todos, alegra-me muito ter podido celebrar e partilhar com vocês tantos momentos importantes.

Aos meus colegas de laboratório, obrigada por todos os momentos de amizade e galhofa que marcaram estes seis anos da minha passagem pelo laboratório de sinapses glutamatérgicas. Cada uma das pessoas que por lá passaram me ensinou alguma coisa. Um agradecimento especial:

Aos gémeos, Aninhas, Grace e Tat obrigada por serem gemini! Obrigada pela amizade e pela parvoíce partilhada. Ajudou-me mais do que possam algum dia imaginar a "sobreviver" ao doutoramento. Ao Rui e à Raquel gostaria de agradecer a boa disposição constante e dizer que foi um prazer conhecer-vos e ter a oportunidade de partilhar convosco algumas das aventuras e desventuras deste doutoramento.

À Miri, o terceiro elemento do gang da OGD, gostaria de agradecer a sua doçura e paciência. Obrigada por toda a ajuda e disponibilidade. Foi um prazer partilhar contigo as "aventuras" da OGD! Ao Rui Costa, colega dos tempos de licenciatura, obrigada pelo companheirismo e ajuda! Um obrigado ao Márcio pela amizade, apesar da distância.

Ao colega Carlos, escute, obrigada pelos momentos de galhofa, muitas vezes por si motivados e iniciados. Foi um prazer trabalhar consigo ao nível do laboratório. Aos preferiti, Pedro Isabel e Diogo, e também ao Ivan e à Domi gostaria de agradecer o humor e a boa disposição que trazem ou trouxeram sempre para o laboratório. Gostei muito de trabalhar e rir com vocês. À Joaninha Pedro gostaria ainda de agradecer a amizade e companheirismo.

À Mãezinha Andrea, ao Paizinho Johnny e ao Costini, foi um prazer conhecer-vos e ter a oportunidade de trabalhar convosco. Obrigada pela amizade. Um viva ao gang da excitotoxicidade.

À Vindy gostaria de agradecer pela amizade, pela boa disposição e, claro, pelo amadrinhamento partilhado da Tuala. Um enorme obrigado a ti e à Fernandinhas por me terem apresentado ao maravilhoso mundo do Doutor.

À D. Céu e à Beta gostaria de agradecer por toda a ajuda no laboratório, sem vocês o caos seria total. Obrigada por ser como uma mãe para todos nós, D. Céu.

Gostaria de agradecer aos meus pais, Rita e Zé, por serem as pessoas maravilhosas que são. Os valores que me transmitiram com tanta naturalidade e sem esforço são os meus maiores bens. O orgulho e amor incondicional que têm por mim fazem-me acreditar que cada pequena vitória minha tem sempre importância e sei que estarão sempre lá para apoiar qualquer rumo que resolva tomar. Por isso gostaria de vos agradecer, por me aceitaram, com todas as minhas qualidades e defeitos sem qualquer tipo de pressão ou julgamento. Se algum dia puder ser uma fracção daquilo que vocês são e me ensinaram, aí serei uma pessoa com missão cumprida.

À minha irmã gostaria de agradecer pela pessoa que ela é e por ser, antes de tudo, minha amiga. O companheirismo e compreensão que sempre partilhámos são muito queridos para mim. À Ana Sofia, que é para mim como uma irmã mais nova, um agradecimento pela sua simplicidade e carinho.

Não podia também deixar de reservar um lugar especial a uma das minhas mais antigas amigas, a Carole. Optámos por rumos semelhantes, por amor à ciência. Por isso e por muito mais que temos partilhado nestes anos de amizade, um enorme obrigado. Obrigada também por aceitares as minhas manias dos concertos e afins e por seres a minha parceira nessas aventuras que já nos deram tanto que falar e rir.

Onde quer que esteja, sei que posso contar sempre com a tua amizade, que é um dos bens mais valiosos que alguma vez poderia ter.

Por fim, gostaria de relembrar a minha avó, que infelizmente já nos deixou. Gostaria de agradecer por ser tudo aquilo que uma criança pode desejar numa avó! A minha infância nunca teria sido a mesma sem o que vivi consigo. Gostaria também de recordar a Marta Filipa. Gostaria muito de poder partilhar este tipo de conquista contigo. A tua prematura partida deste mundo marcou muito a minha personalidade e deixou para sempre uma saudade que nunca poderá ser colmatada. Mas apesar disso, a tua lembrança, trago-a sempre junto a mim.

Não posso deixar de aproveitar a ocasião para referir os meus gatinhos, Pretinho, Branquinha e claro, Ervilhinha. O vosso carinho descomprometido é muito importante para mim, pois é algo inteiramente puro e desinteressado. Obrigado por isso.

Gostaria de dedicar esta tese à minha família e amigos por acreditarem sempre em mim e por estarem sempre do meu lado. Muito obrigada.

"In the dark, see past our eyes
Pursuit of truth no matter where it lies
Gazing up to the breeze of the heavens
On a quest, meaning, reason"
(*Through the Never*, Hetfield, Ulrich & Hammet)

Table of Contents

Abbreviations

24S-OHC	24(S)-Hydroxycholesterol
Actb	β-Actin
AD	Alzheimer's Disease
AIF	Apoptosis-inducing Factor
AIP	Apaf-1 Interacting Protein
ALS	Amyotrophic Lateral Sclerosis
AMPAR	α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid Receptor
ANOVA	One-way Analysis of Variance
AP-1	Activator Protein-1
AP2	Clathrin Adaptor Protein
Apaf-1	Apoptotic Peptidase Activating Factor 1
ASIC	Acid-sensing Ion Channel
ASK	Apoptosis Signal Regulating Kinase
ATD	Amino Terminal Domain
ATF	Activating Transcription Factor
Bcl	B-cell Lymphoma
BDNF	Brain-derived Neurotrophic Factor
BID	BH3 Interacting-domain Death Agonist
BNip3	BCL2/adenovirus E1B 19 kDa Interacting Protein 3
BSA	Bovine Serum Albumin
CA	Cornu Ammonis
CAD	Caspase Activated DNase
CaMK	Ca^{2+}-Calmodulin Kinase
CBP	CREB Binding Protein
CD95	Cluster of Differentiation 95
Cdk5	Cyclin-dependent Kinase 5
cIAP	Cellular Inhibitor of Apoptosis Protein
CK2	Casein Kinase 2
CLAP	Chymostatin, Leupeptin, Antipain and Pepstatin
CNS	Central Nervous System
CP-AMPAR	Ca^{2+}-permeable AMPAR
CREB	cAMP Response Element-binding Protein
C_t	Threshold Cycle
CTD	Carboxyl Terminal Domain
Ctx	Cerebral Cortex

CYLD	Cylindromatosis
DD	Death Domain
DED	Death Effector Domain
DG	Dentate Gyrus
DISC	Death Inducing Signaling Complex
DIV	Days *in vitro*
DL	Death Ligand
DN	Dominant Negative
DR	Death Receptor
Drp-1	Dynamin Related Protein 1
DUB	Deubiquitinase
E18	Embryonic Day 18
ECF	Enhanced Chemifluorescence
EDD	E3 Ubiquitin-protein Ligase UBR5
ER	Endoplasmic Reticulum
ERK	Extracellular-signal Regulating Kinase
FADD	Fas-associated Protein with Death Domain
FasL	Fas Ligand
FL	Full Length
FLIP	FLICE-like Inhibitory Protein
FOXO	Forkhead O
GADD45	Growth Arrest and DNA Damage 45
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GLUD1	Glutamate Dehydrogenase 1
GLUL	Glutamate-ammonia Ligase
GluR	Glutamate Receptor
H2AX	H2A Histone Family, Member X
HBSS	Hank's Balanced Salt Solution
HD	Huntington's Disease
HtrA2	High Temperature Requirement Protein A2
ICAD	Inhibitor of CAD
ID	Intermediate Domain
IFNγ	Interferon Gamma
iGluR	Ionotropic GluR
IκB	Inhibitor of Kappa B
IKK	IkB Kinase

IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
JBD	JNK Binding Domain
JIP	JNK-interacting Protein
JNK	c-Jun N-terminal Kinase
KAR	Kainate Receptor
KD	Knock-down
KO	Knock-out
LBD	Ligand Binding Domain
LDH	Lactate Dehydrogenase
LTD	Long-term Depression
LTP	Long-term Potentiation
LUBAC	Linear Ub Chain Assembly Complex
MADD	MAP-kinase Activating Death Domain
MAGUK	Membrane Associated Guanylate Kinase
MAPK	Mitogen-activated Protein Kinase
MAPKAPK2	MAPK-activated Protein Kinase 2
MAPKK	MAPK Kinase
MAPKKK	MAPK Kinase Kinase
MCAO	Middle Cerebral Artery Occlusion
MCMV	Mouse Cytomegalovirus
MEF	Mouse Embryonic Fibroblast
MEK	MAPK/ERK Kinase
MEKK	MEK Kinase
MEM	Minimal Essential Medium
mGluR	Metabotropic GluR
MK-801	(5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine Maleate
MKP	MAPK Phosphatase
MLK	Mixed-lineage Kinase
MLKL	Mixed-lineage Kinase Domain-like
MNK	MAPK Interacting Kinase
MS	Multiple Sclerosis
MSK	Mitogen and Stress Activated Kinase
MW	Multiwell
NCX	Na^{+}/Ca^{2+} Exchanger

Nec-1	Necrostatin-1
NEMO	NF-kB Essential Modulator
NF-kB	Nuclear Factor Kappa B
NGF	Nerve Growth Factor
NHE1	Na^+/H^+ Exchanger 1
NMDAR	N-methyl-D-aspartate Receptor
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOS1AP	NOS1 Associated Protein
OGD	Oxygen and Glucose Deprivation
OSM	Osmosensing Scaffold for MEKK3
PARP	Poly-ADP Ribose Polymerase
PBS	Phosphate-buffered Saline
PD	Parkinson's Disease
PDL	Poly-D-lysine
PDZ	PSD95/Dlg/ZO-1
PGAM5	Serine/threonine-protein Phosphatase
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PK	Protein Kinase
PMSF	Phenylmethanesulfonyl Fluoride
POSH	Plenty of SH3s
PP	Protein Phosphatase
PSD	Post-synaptic Density
PVDF	Polyvinylidene Fluoride
PYGL	Phosphorylase, Glycogen, Liver
qRT-PCR	Quantitative Real-time PCR
RHIM	RIP Homotypic Interaction Motif
RIP	Receptor-interacting Protein Kinase
ROS	Reactive Oxygen Species
RSK	p90 Ribosomal S6 Kinase
SAP	Synapse-associated Protein
SAPK	Stress-activated Protein Kinase
SBDP	α-Spectrin Break-down Product
shRNA	Short Hairpin RNA
SIRT2	Sirtuin2

Smac/	Second Mitochondria-derived Activator of Caspase/
DIABLO	Direct Inhibitor of Apoptosis-binding Protein with Low pI
Sp-1	Specificity Protein 1
STEP	Striatal-Enriched Tyrosine Phosphatase
TAB	TAK1 Binding Protein
TACE	TNFα Converting Enzyme
TAK1	Transforming Growth Factor-β Activated Kinase-1
TAOK	TAO Kinase
TBI	Traumatic Brain Injury
TBS-T	Tris-buffered Saline-Tween
TLR	Toll-like Receptor
TNFR1	Tumor Necrosis Factor Receptor 1
TNFα	Tumor Necrosis Factor α
TORC	Transducer of Regulated CREB Activity
tPA	Tissue Plasminogen Activator
TPL2	Tumor Progression Locus 2
TRADD	TNFR-associated via Death Domain
TRAF2	TNFR Associated Factor 2
TRAIL	TNF-related Apoptosis-inducing Ligand
TRP	Transient Receptor Potential
TWEAK	TNF-related Weak Inducer of Apoptosis
VO	Vessel Occlusion
WT	Wild-type
zVAD.fmk	N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me)-fluoromethyl Ketone

Resumo

A isquemia cerebral induz neurodegeneração de populações específicas de neurónios, nomeadamente na área CA1 do hipocampo. Apesar da sua elevada prevalência e dos esforços na investigação, não existem actualmente tratamentos eficazes para prevenir a neurodegeneração associada à isquemia global. Esta patologia pode ser estudada *in vitro* submetendo os neurónios a privação de oxigénio e glicose (OGD, do inglês "oxygen and glucose deprivation"). O principal objectivo deste trabalho foi o de estudar os mecanismos moleculares associados à morte neuronal iniciada pela OGD em culturas primárias de neurónios de hipocampo. Para este propósito pesquisámos diferentes aspectos da morte celular. Assim, estudámos o fenómeno de excitotoxicidade mediada por receptores de glutamato do tipo NMDA (NMDARs), analisámos a activação de um novo mecanismo de morte celular, necroptose, e verificámos a activação de vias efectoras da morte celular, em particular, as vias das MAPKs.

Começámos por estudar a influência da subunidade GluN2B dos NMDARs na morte neuronal induzida por OGD. Os NMDARs têm um papel de relevo no excesso de Ca^{2+} intracelular característico da excitotoxicidade; contudo, o papel das subunidades GluN2 tem permanecido controverso. Há evidências que mostram o envolvimento das subunidades GluN2A e GluN2B no processo de morte celular enquanto outras mostram o papel tóxico do GluN2B e uma função neuroprotectora do GluN2A. Para clarificar esta questão recorremos a um sistema de cultura neuronal de murganhos GluN2B$^{-/-}$ e observámos um papel determinante da subunidade GluN2B na indução de morte após OGD em neurónios de córtex. Verificámos que a ausência do GluN2B eliminou a toxicidade induzida por OGD, que foi recuperada com a reintrodução da subunidade nos neurónios GluN2B$^{-/-}$. Demonstrámos ainda o papel preponderante do domínio C-terminal (CTD) da subunidade na toxicidade mediada por GluN2B e mapeámos alguns locais de interacção no CTD de GluN2B responsáveis por esta função tóxica. O domínio PDZ é responsável pela interacção com a PSD95, que faz o acoplamento à nNOS. A interferência com esta interacção teve um efeito neuroprotector. Identificámos também dois determinantes moleculares no CTD de GluN2B relevantes para este processo, o local de ligação às proteínas AP2 e CaMKII. A mutação destes locais eliminou a toxicidade induzida pela subunidade GluN2B. Estas evidências apoiam o papel determinante dos NMDARs contendo GluN2B num contexto de isquemia cerebral *in vitro*. O nosso estudo é particularmente relevante na

medida em que os trabalhos anteriores utilizavam, na sua maioria, estímulos excitotóxicos.

De seguida, investigámos a indução de morte neuronal por necroptose, um novo mecanismo de necrose programada, em neurónios de hipocampo submetidos a OGD. Este tipo de morte celular ocorre na sequência da activação de "death receptors" (DRs). Em determinadas condições, ocorre a formação de um complexo que medeia a apoptose e inibe a necroptose através da clivagem das proteínas RIP1/3. Contudo, se ocorrer inibição da caspase8, a RIP3 é recrutada para a RIP1 e juntas formam um complexo chamado necrosoma, que activa a necroptose. A OGD induziu um componente de morte celular que foi revertido pelo inibidor de necroptose Nec-1, mas não pelo inibidor da apoptose zVAD.fmk. A especificidade do efeito protector da Nec-1 sobre a necroptose foi comprovada pela ausência de efeito da Nec-1 no componente apoptótico. Além disso, a OGD induziu a expressão das proteínas RIP1 e RIP3. Confirmámos o papel tóxico da RIP3 através de ensaios de sobreexpressão e silenciamento. A sobreexpressão das proteínas RIP1 e RIP3 aumentou a morte neuronal, enquanto o silenciamento da RIP3 reduziu a toxicidade induzida por OGD. O efeito tóxico da OGD foi recuperado com a reintrodução da proteína RIP3 em neurónios sem RIP3 endógena. Por fim, relacionámos os mecanismos observados *in vitro* com o modelo *in vivo*, ao observarmos que a isquemia global induz o aumento da expressão da RIP3 na área CA1 do hipocampo.

Estudámos por fim a activação de MAPKs em neurónios de hipocampo submetidos a OGD. Estas cinases são responsáveis pela resposta ao stress celular e estão envolvidas em diversos paradigmas de morte neuronal. Verificámos a activação das cinases p38 e JNK após OGD, às 2h e 6h de recuperação, respectivamente. Verificámos ainda que a inibição destas cinases teve um efeito protector, o que sugere um papel citotóxico. Curiosamente, a cinase JNK parece ter um duplo papel, já que a sua inibição só foi protectora quando efectuada às 4h após o estímulo de OGD.

Concluindo, os nossos resultados demonstram que a OGD afecta os neurónios a vários níveis, incluindo na indução de mecanismos que contribuem para a neurodegeneração. Este modelo *in vitro* apresenta-se assim como uma ferramenta importante para a dissecção de mecanismos moleculares subjacentes à isquemia cerebral, o que poderá contribuir para o desenvolvimento de estratégias terapêuticas para esta patologia.

Abstract

Cerebral global ischemia induces selective neurodegeneration of specific subsets of neurons throughout the brain, namely in the CA1 region of the hippocampus. Despite its high prevalence and intensive research, there is still need of effective treatments to reduce the neurodegeneration associated with global ischemia. This pathology can be studied *in vitro* by depriving neurons of oxygen and glucose (OGD). Our main goal was to study the molecular mechanisms of neuronal death activated by OGD in primary cultures of hippocampal neurons. For this purpose we targeted distinct aspects of cell death. We studied the excitotoxic component of cell death mediated by NMDARs, we addressed the activation of a novel mechanism of programmed cell death, necroptosis, and we analyzed the activation of effector signaling cascades, in particular MAPKs.

We started by studying the influence of the GluN2B subunit of NMDARs to OGD-induced neuronal demise. NMDARs are major contributors to the overload of intracellular Ca^{2+} characteristic of excitotoxicity and the role of GluN2 subunits has remained controversial. This is due to a variety of conflicting evidence showing that either both GluN2A and GluN2B contribute to neuronal death or that GluN2B is mostly pro-death and GluN2A pro-survival. To clarify this question we used cultured cortical neurons from GluN2B$^{-/-}$ mice and wild-type littermates, and observed that GluN2B is determinant for induction of excitotoxic neuronal death following OGD. We observed that the absence of this subunit blocked neuronal death induced by OGD and that the toxicity was rescued when we reintroduced the subunit in the KO neurons. Moreover, we demonstrated that the C-terminal domain (CTD) had a preponderant role in GluN2B-induced toxicity, and we identified molecular determinants in the CTD of GluN2B responsible for this function. We confirmed that the PDZ-binding domain was partly responsible for NMDAR toxicity. This domain is responsible for the interaction with PSD95 that couples to nNOS, and interfering with this interaction was neuroprotective. Additionally, we identified two other regions on the GluN2B CTD that are required for OGD-induced cell death, the AP2- and the CaMKII-binding domains. Mutations in either of these sites blocked GluN2B-mediated toxicity. These findings confirmed the crucial role of GluN2B-containing NMDARs in a context of *in vitro* ischemia, and our study is particularly relevant since most previous work was performed under excitotoxic conditions.

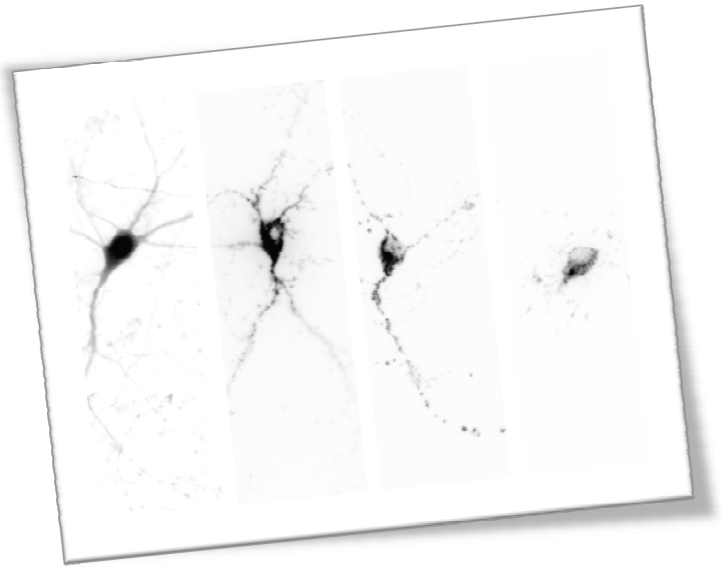
Next, we investigated whether OGD induced necroptosis, a novel type of programmed necrosis, in hippocampal neurons. This type of cell death has been recently described to occur following death receptor (DR) signaling. In certain conditions a complex called DISC is formed. DISC induces apoptosis and downregulates necroptosis via caspase8-mediated cleavage of the proteins RIP1/RIP3. However, when caspase8 is inhibited, RIP3 is recruited to RIP1 and together they form a complex called the necrosome, and activate necroptosis. We observed that OGD induced a component of cell death that was reversed by the necroptotic inhibitor Nec-1 but not by zVAD.fmk, an apoptotic inhibitor. Notably, we observed that Nec-1 had no effect on the apoptotic component of neuronal death. Additionally, OGD induced the expression of RIP1 and RIP3. We confirmed the toxic role of RIP3 by performing overexpression and knock-down experiments. We observed that overexpression of both RIP1 and RIP3 exacerbated neuronal death induced by OGD whereas knockdown of RIP3 significantly reduced OGD-mediated toxicity. The damaging effect of the OGD challenge was rescued by reintroducing RIP3 in neurons where endogenous RIP3 was knocked-down, confirming the specificity of the requirement for RIP3. Finally, we correlated these *in vitro* events with the *in vivo* challenge, by confirming that global cerebral ischemia in the rat also induces RIP3 expression in the CA1 area of the hippocampus.

Lastly, we studied the activation of MAPKs in hippocampal neurons submitted to OGD. These kinases are responsible for the majority of the cellular response to stress and are involved in several paradigms of cell death, including in neurons. We determined that both p38 and JNK are activated following OGD, at 2h and 6h of reoxygenation, respectively. Furthermore, inhibition of the activity of these MAPKs has a neuroprotective effect, suggesting a cytotoxic function. Interestingly, JNK seems to have biphasic function since neuroprotection was only achieved when we inhibited JNK at 4h reoxygenation.

Overall, our results demonstrate that OGD induces a variety of changes in neurons, including several mechanisms that contribute to neurodegeneration. This *in vitro* model is thus a powerful tool to address the molecular mechanisms underlying cerebral ischemia, which may provide useful insights into the development of therapeutic strategies to this pathology.

Chapter I

Introduction



Cerebral Ischemia

Cerebral ischemia is a leading cause of disability and death in the western world (Flynn et al., 2008). This pathology lacks effective treatments, a direct consequence of a limited time window for intervention before major neurodegeneration ensues as well as of the lack of specificity of some of the treatments developed so far. Additionally, the inhibition of some molecular targets proves inefficient due to secondary effects. To date, the only treatment administered to cerebral ischemic patients is tissue plasminogen activator (tPA) (Fonarow et al., 2011; Iadecola and Anrather, 2011b). Cerebral ischemia is broadly divided in two types: global cerebral ischemia and focal ischemia (commonly referred to as stroke) (Flynn et al., 2008).

Transient global ischemia results from a transient interruption of blood supply to the entire brain, due to a cardiac arrest or to a near-drowning situation. This type of pathology causes selective degeneration of specific subsets of neurons throughout the brain, namely the neurons of the Cornu Ammonis 1 (CA1) region of the hippocampus, the cortical neurons of layers II, V and VI, the Purkinje cells of the cerebellum and the dorsolateral striatal neurons (Lo et al., 2003; Zukin et al., 2004).

Focal ischemia arises from the occlusion of a blood vessel in the brain (ischemic stroke) or from the rupture of a brain vessel (hemorrhagic stroke) leading to the deprivation of blood flow on the region of the brain supplied by that vessel. The area most severely affected by hypoperfusion constitutes the infarct core, in which most neurons degenerate due to an overwhelming stress, and the surrounding area, the penumbra, has a gradient of perfusion and mixed neuronal phenotypes, with some cells dying whilst others are able to survive (Kunz and Iadecola, 2009).

The interruption in the blood supply to the brain causes a deprivation of oxygen and glucose, leading to a failure in ATP production, which is pernicious due to the brain high metabolic demand (Moskowitz et al., 2010). This results in neuronal depolarization and excessive glutamate release, accompanied by dysfunction of the glutamate reuptake mechanisms in glia cells and neurons, as a result of the dissipation of the Na^+ gradient, due to the depletion of ATP. Thus, an excessive glutamate accumulation at the synapse causes the overactivation of glutamate receptors leading to an intracellular Ca^{2+} overload that may trigger cytotoxicity, a phenomenon called excitotoxicity. Notably, depending on the duration and intensity of the insult, other non-excitotoxic mechanisms are activated, contributing to Ca^{2+} overload (Besancon et al., 2008; Szydlowska and Tymianski, 2010). Deregulation of Ca^{2+} homeostasis activates

deleterious intracellular mechanisms, including mitochondrial dysfunction and oxidative and nitrosative stress, that induce inflammation and ultimately neuronal death (Figure 1.1) (Moskowitz et al., 2010; Iadecola and Anrather, 2011a). In global ischemia and in the penumbra area, neuronal degeneration ensues within 24-72h after the ischemic insult, a process called delayed neuronal death. This delay in neuronal demise onset suggests the reliance on transcriptional changes, leading to programmed cell death mechanisms.

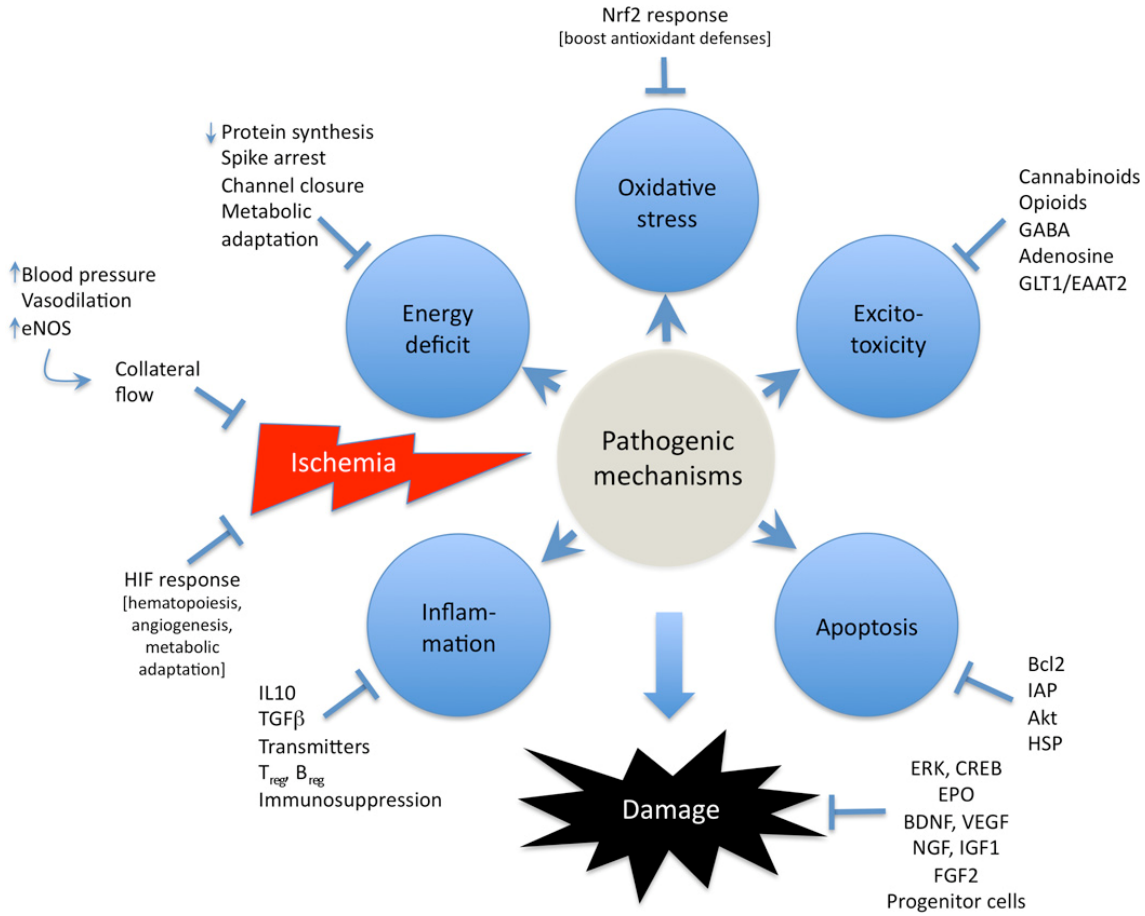


Figure 1.1. Cerebral ischemia induces neuronal demise via activation of deleterious mechanisms. Upon interruption of blood flow, distinct damaging mechanisms are activated that contribute to the injury. In an early phase, energy imbalance and excitotoxicity are activated contributing to mounting oxidative and nitrosative stress. These contribute to inflammatory signaling and ultimately programmed cell death mechanisms are activated. Concomitantly, several neuroprotective mechanisms are activated, to counteract the effect of these damaging signals. (Iadecola and Anrather, 2011b)

Cerebral ischemia can be addressed experimentally, using several research models, both *in vitro* and *in vivo*. The most common *in vivo* models to study transient global ischemia are the four vessel occlusion (4-VO) and the 2-VO combined with hypotension, both in the rat. As for focal ischemia, the middle cerebral artery occlusion (MCAO) model is generally used. Regarding the *in vitro* models, the closest to *in vivo* ischemia is the oxygen and glucose deprivation (OGD) challenge, which is most commonly used either in primary cultures of hippocampal or cortical neurons or in forebrain or organotypic hippocampal slices. Due to its characteristics, the OGD challenge of hippocampal neurons is a good model for global ischemia and allows for the dissection of molecular pathways underlying cerebral ischemia. This challenge consists of placing the neurons or slices in a glucose-free medium inside an anaerobic chamber, thereby combining the deprivation of these two factors which mimics what happens in the brain during the interruption of the blood flow, but in a simplified system (Zukin et al., 2004). This model also has advantage over other *in vitro* models [for example, application of high concentrations of glutamate receptor (GluR) agonists] since it accounts for most changes observed during blood supply deprivation, like activation of non-excitotoxic mechanisms, such as Ca^{2+} entry via acid-sensing ion channels (ASICs), transient receptor potential (TRP) channels or Na^{+}/Ca^{2+} exchangers (NCXs) (Szydlowska and Tymianski, 2010), instead of accounting only for excitotoxic mechanisms.

EXCITOTOXICITY

The term excitotoxicity was first used by Olney (1969) to describe neurodegeneration associated with the activation of excitatory amino acid receptors (Olney, 1969). This phenomenon is a hallmark of several neurodegenerative disorders, such as Alzheimer's disease (AD), Huntington's disease (HD) and cerebral ischemia, and it results from excessive glutamate release from the presynaptic terminal, due to the specific disease-associated insult (Figure 1.2). This abnormal glutamate release combined with the dysfunction of the mechanisms responsible for removing this neurotransmitter from the synapse result in the accumulation of glutamate in the synaptic cleft, for longer periods of time. The immediate consequence of this accumulation is the overactivation of GluRs, present at the postsynaptic membrane, leading to a massive influx of ions, namely Ca^{2+} (Choi, 1987; Choi et al., 1987; Mehta et al., 2013).

Calcium acts physiologically as a second messenger in neurons, which possess highly specialized mechanisms of Ca^{2+} buffering to maintain this ion at low concentrations. Thus, localized increases in Ca^{2+} concentration lead to the activation of specific intracellular signaling pathways. Upon an excitotoxic insult, the Ca^{2+} overload induces the deregulation of Ca^{2+} buffering mechanisms, for exceeding their capacity, contributing to the activation of damaging Ca^{2+}-dependent processes that may ultimately lead to neuronal death (Choi, 1988; Tymianski and Tator, 1996; Arundine and Tymianski, 2003; Mehta et al., 2013). Evidence linking excessive Ca^{2+} influx to neuronal damage lead to the formulation of the "Calcium Hypothesis" which states that "neuronal calcium overload contributes to neurodegeneration" (Arundine and Tymianski, 2003).

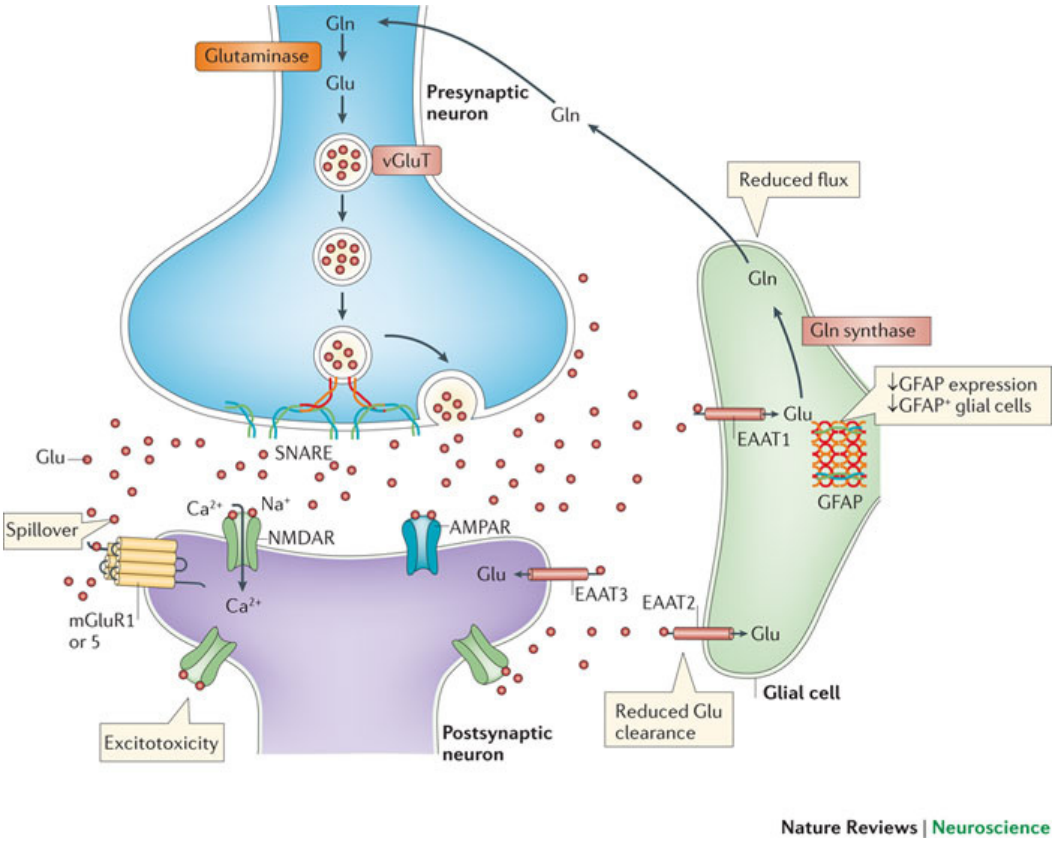


Figure 1.2. Mechanisms of excitotoxicity. The excessive glutamate release from the presynaptic terminal combined with the dysfunction of the glial mechanisms of glutamate clearance result in excessive concentrations of glutamate in the synapse. This leads to overactivation of GluRs located postsynaptically and activation of pro-death signaling pathways that promote neuronal demise. (Popoli et al., 2012)

The understanding of the paramount role exerted by Ca^{2+} to the mechanisms of excitotoxicity lead to initial efforts aiming at the blockade of this Ca^{2+} overload, to prevent neurodegeneration. This strategy, however, proved ineffective since GluR antagonists also blocked their physiological functions, which had adverse side effects (Lees, 1998). Thus, researchers started focusing on the intracellular pathways that are activated as a consequence of Ca^{2+} overload. Notably, the route of Ca^{2+} entry seems to be determinant for the activation of specific downstream signaling pathways that have distinct contributions to neuronal fate ("Source-specificity Hypothesis") (Bading et al., 1993; Ghosh and Greenberg, 1995; Sattler and Tymianski, 2001). Due to their role as primary gateways for Ca^{2+} entry in neurons, ionotropic GluRs (iGluRs) are one of the main sources of neuronal Ca^{2+}, in particular N-methyl-D-aspartate receptors (NMDARs), which are highly permeable to this ion (Mehta et al., 2013).

Glutamatergic Neurotransmission

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS). Glutamate is released from the presynaptic terminal, during excitatory neurotransmission, leading to its diffusion across the synaptic cleft and consequent activation of GluRs present at the postsynaptic membrane (Catarzi et al., 2007; Greger et al., 2007; Hansen et al., 2007). Glutamatergic synapses undergo activity-dependent long-lasting changes in synaptic strength, such as long-term potentiation (LTP) and long-term depression (LTD), mechanisms that are necessary for learning and memory (Malenka and Nicoll, 1999; Catarzi et al., 2007; Greger et al., 2007).

Glutamate activates two major classes of receptors: metabotropic GluRs (mGluRs) and ionotropic GluRs. The mGluRs mediate slow synaptic responses, due to their coupling to intracellular G proteins. To date, eight subtypes of mGluR family members have been identified, mGluR1-8, and classified into three groups (groups I–III), based on sequence similarity, pharmacology and transduction mechanism (Friedman, 2006; Catarzi et al., 2007). The iGluRs are responsible for the majority of fast excitatory neurotransmission in the mammalian brain. These are heterotetrameric cation channels, comprising three functionally distinct subtypes: α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainate (KA) and NMDA receptors. These receptors are mainly concentrated at postsynaptic sites, where they fulfill a variety of

different functions (Cull-Candy et al., 2006; Greger et al., 2007). The NMDARs mediate the slow component of excitatory postsynaptic currents. NMDARs are highly permeable to Ca^{2+}, Na^{+} and K^{+} and can be fully activated under membrane depolarization, which is necessary to abolish the inhibition of the receptor by Mg^{2+}. The membrane depolarization is mainly afforded by AMPAR activation, which mediates the fast component of excitatory neurotransmission. Most AMPARs, as well as the KARs, are permeable to Na^{+} and K^{+}, but impermeable to Ca^{2+}. The permeability of AMPARs to Ca^{2+} is dependent on their subunit comsition and is regulated by the GluA2 subunit (Won et al., 2002; Hazell, 2007).

Eukaryotic iGluR subunits are each composed of an extracellular amino-terminal domain, a ligand binding domain (LBD), a transmembrane domain, and an intracellular carboxy-terminal domain (Figure 1.3). The transmembrane domain contains three membrane-spanning segments (M1, M3 and M4) and a ‘‘re-entrant loop’’, termed M2, which is located at the pore-lining region of the receptor, as determined by crystallography (Sobolevsky et al., 2009). Two extracellular segments, S1 and S2, have been shown to constitute the LBD which is responsible for the binding of both the neurotransmitter and the competitive agonists/antagonists. Glutamate binding to the extracellular LBD segment of the receptor triggers a series of conformational changes that lead to activation of the receptor (Catarzi et al., 2007).

One of the goals of this work consisted of studying the contribution of NMDAR subunits for OGD-induced death. For that reason, this introductory section will be mainly focused on NMDARs.

NMDA RECEPTORS

The NMDARs are heterotetrameric structures composed of different combinations of three types of subunits: GluN1, GluN2 and GluN3 (Hollmann and Heinemann, 1994; Wenthold et al., 2003). These receptors are most commonly assembled from two GluN1 subunits and two GluN2 subunits. NMDARs containing GluN1 and GluN2 subunits are ion channels with high permeability to Ca^{2+} (MacDermott et al., 1986; Mayer and Westbrook, 1987), which contributes to their chief role in the mechanisms of excitotoxicity. In order to be activated, NMDARs require binding of glutamate and also glycine (or D-serine), which are mandatory cofactors (Johnson and Ascher, 1987; Labrie and Roder, 2010). Agonist binding combined with membrane depolarization contributes to relief of the Mg^{2+} block and channel opening

(Mayer et al., 1984; Nowak et al., 1984). The activity of NMDARs is modulated by polyamines (for example, spermine) (Benveniste and Mayer, 1993; Rock and Macdonald, 1995), among other factors. The NMDARs have paramount roles in physiological processes, such as synaptic plasticity, learning and memory but also in diverse neuropathologies, including AD, schizophrenia and cerebral ischemia (Lau and Zukin, 2007).

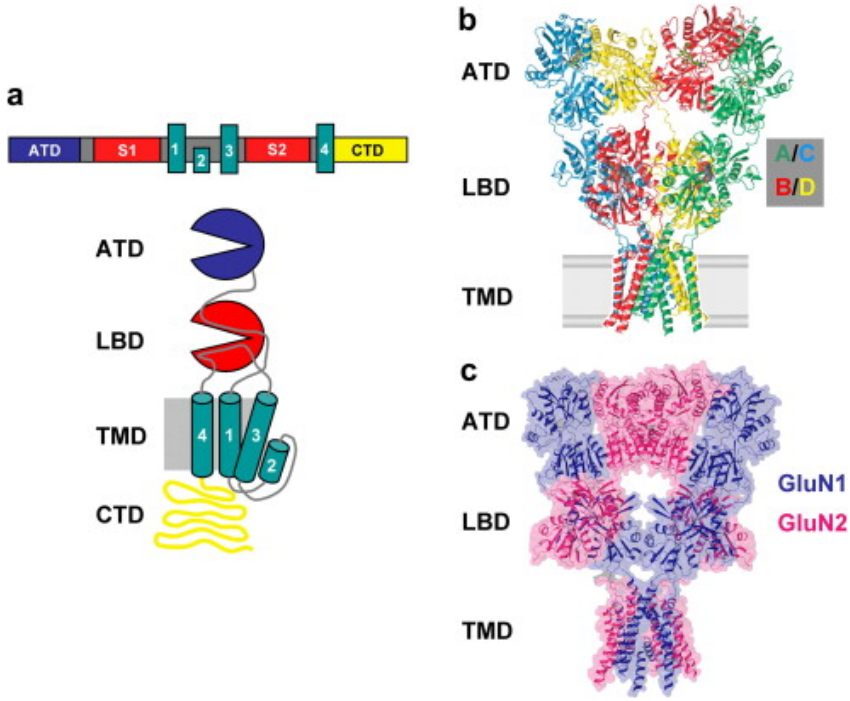


Figure 1.3. iGluR subunit structure. (A) iGluR subunits possess four functional domains: the extracellular amino-terminal domain (ATD) and LBD, the transmembrane domains comprising three membrane-spanning helices (M1, M3 and M4) and the re-entrant P-loop region of M2 and the large intracellular C-terminal domain (CTD). **(B)** Ribbon structure representation of the rat GluA2 homomeric AMPAR with each of the four subunits coloured coded and indicating the two conformationally distinct pairs of subunits, A/C and B/D. **(C)** Model NMDAR structure based on the GluA2 crystal structures of the ATD and transmembrane domains and the GluN1-GluN2A LBD heterodimer crystal structure. (Adapted from Sobolevsky et al., 2009; Wyllie et al., 2013)

NMDAR Subunits

The GluN1 is an obligatory subunit that binds glycine or D-serine instead of glutamate (Fukaya et al., 2003; Furukawa and Gouaux, 2003). This subunit has 8 splice variants that may modulate synaptic targeting of the receptors (Ferreira et al., 2011). Changes in ambient pH can modulate NMDAR function as protons can exert an

inhibitory effect on NMDARs via direct interaction with a Lys residue on GluN1 subunits (Traynelis and Cull-Candy, 1990). GluN1 subunits and the level of neuronal activity are two contributing factors that regulate the exit of NMDARs from the endoplasmic reticulum (ER). These subunits are produced in large excess relatively to GluN2 subunits (Huh and Wenthold, 1999; Mu et al., 2003) and are retained in the ER until they are assembled with other subunits (Wenthold et al., 2003). Upon assembly of functional receptors, vesicles containing NMDARs are transported to dendrites. This is a type of active transport process, relying on motor proteins that carry the vesicles along microtubules (Hirokawa and Takemura, 2004).

The GluN2 subunits can be divided in four subtypes, GluN2A-D. These four subunits have distinct expression in neurons: GluN2A is widely distributed throughout the brain, while GluN2B is predominantly expressed in the forebrain, GluN2C in the cerebellum and GluN2D is mostly found in the thalamus (Buller et al., 1994). Thus, the most common assemblies of NMDARs consist of GluN1-GluN2A receptors or GluN1-GluN2B (Al-Hallaq et al., 2007), but triheteromeric receptors also exist (Sheng et al., 1994; Cull-Candy and Leszkiewicz, 2004; Brothwell et al., 2008). Different subunit compositions fine-tune the receptors, namely in terms of intracellular interactions, localization in the membrane, and also channel properties (Figure 1.4). Indeed, relatively to GluN2B, the GluN2A subunit confers the receptor faster kinetics, lower glutamate affinity, greater channel open probability and more prominent Ca^{2+}-dependent desensitization. Moreover, the GluN2C/D subunits confer reduced sensitivity to the Mg^{2+} block and lower conductance opening (Cull-Candy and Leszkiewicz, 2004; Wyllie et al., 2013).

There are two subtypes of GluN3 subunits, GluN3A and GluN3B. The former are expressed throughout the CNS, while the latter are found mostly in motor neurons. Like GluN1, these subunits bind glycine (Yao et al., 2008). When present in the final receptor assembly, GluN3 subunits confer lower Ca^{2+} permeability and reduced surface expression (Cull-Candy and Leszkiewicz, 2004).

The GluN2 subunits share high degree of similarity in their extracellular and transmembrane domains, suggesting a common function for these domains in all subunits. Notably, the C-terminal domain of NMDARs is the most divergent region among the subunits. In fact, the GluN2A and GluN2B subunits share only 29% identity in their C-terminal domains. This region enables the NMDARs to interact with diverse intracellular proteins and links the receptor to downstream signaling pathways. The

divergent C-terminus among the different subunits suggests functional adaptations in intracellular signaling, specific for each GluN2 subunit (Ryan et al., 2008).

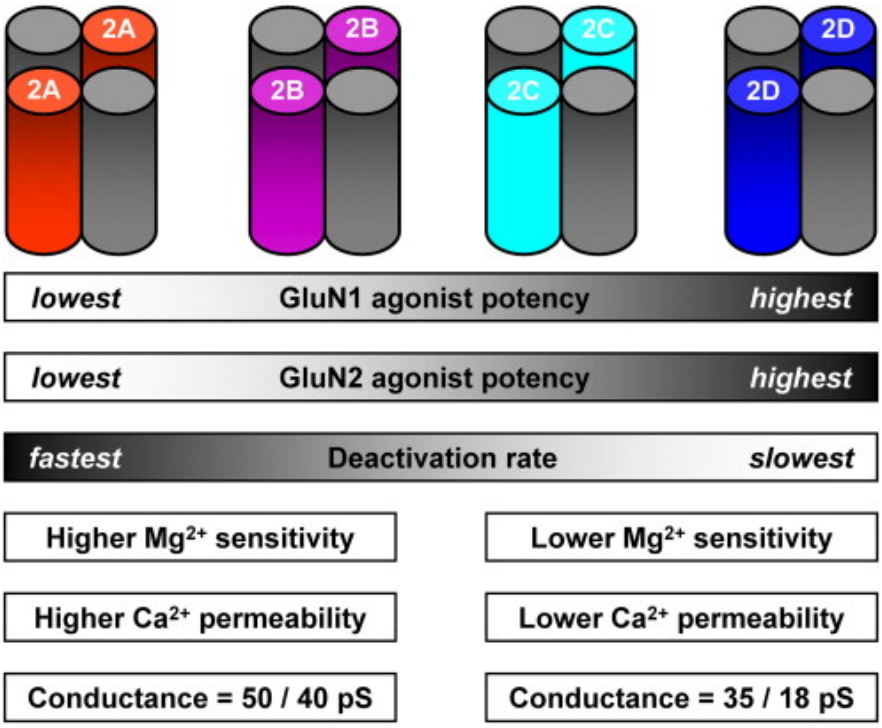


Figure 1.4. The subunit composition of NMDARs influences channel properties. The four di-heteromeric NMDARs (GluN1 subunits indicated in grey) display different features, such as agonist and co-agonist potency (lowest – GluN1/GluN2A NMDARs, highest – GluN1/GluN2D NMDARs); deactivation rates (fastest – GluN2A-containing NMDARs, slowest – GluN2D-containing NMDARs); voltage-dependent block by Mg^{2+}; permeability to Ca^{2+} and unitary conductance. (Wyllie et al., 2013)

NMDAR Regulation by Phosphorylation

One of the key mechanisms that regulate iGluR function and localization is phosphorylation (Lee, 2006). Accordingly, the C-terminal domain of NMDARs, particularly of the GluN2A and GluN2B subunits, has diverse sites of phosphorylation (Chen and Roche, 2007). Several kinases, including Ca^{2+}-calmodulin kinase II (CaMKII), cyclin-dependent kinase 5 (Cdk5), Src, protein kinase A (Tararuk et al.), PKC and casein kinase 2 (CK2) have the ability to phosphorylate NMDAR subunits, with different impact on channel properties and trafficking of the receptors (Chen and Roche, 2007). The protein CaMKII is a Ca^{2+}-dependent kinase that autophosphorylates at Thr286 as a consequence of NMDAR activation, promoting its translocation to the spines where it interacts with GluN2B subunits (Shen and Meyer, 1999; Bayer et al.,

2006). This kinase shows higher affinity for GluN2B subunits than GluN2A (Strack and Colbran, 1998). CaMKII activity regulates synaptic plasticity phenomena via phosphorylation of AMPARs (Lisman et al., 2002) and modulation of NMDAR function (Gardoni et al., 2001; Gardoni et al., 2003; Chung et al., 2004).

Influence of GluN2 Subunits on Neuronal Fate

GluN2A and GluN2B are believed to have differential contributions to excitotoxic neuronal death (Wyllie et al., 2013). Indeed, while GluN2A is considered to have a prosurvival role (Liu et al., 2007; Terasaki et al., 2010), GluN2B overactivation is thought to exert a detrimental effect (Aarts et al., 2002; Soriano et al., 2008; Martel et al., 2012). However, there are also some reports that both subunits may contribute to neuronal death (Graham et al., 1992; Stanika et al., 2009; Zhou et al., 2013), suggesting that the regulation of these mechanisms implies an interplay of factors, such as subunit composition and receptor localization (Lai et al., 2011). Indeed, although both GluN2A and GluN2B are located at synaptic and extrasynaptic sites (Thomas et al., 2006; Harris and Pettit, 2007; Zhou et al., 2013), GluN2A is thought to preferentially locate in the synapse and GluN2B is believed to concentrate extrasynaptically (Kew et al., 1998; Li et al., 1998b; Scimemi et al., 2004; Groc et al., 2006; Zhang and Diamond, 2009).

The C-terminal domain of GluN2 subunits seems to be determinant for NMDAR-mediated toxicity (Martel et al., 2012). This report elegantly demonstrates that the influence of chimeric GluN2 subunits to neuronal death relies on the identity of the C-terminal domain. Swapping the C-terminal domain of GluN2A for that of GluN2B promotes neuronal demise in an excitotoxic context (Figure 1.5). The complementary experiment, swapping the C-terminal domain of GluN2B for that of GluN2A does the opposite, promoting neuronal survival (Cepeda and Levine, 2012; Martel et al., 2012). Additional support of the concept of a detrimental role of the GluN2B subunit is advanced by several reports demonstrating a neuroprotective effect of interfering with activation of downstream signaling pathways, specifically neuronal nitric oxide synthase (nNOS) (Aarts et al., 2002; Cui et al., 2007a; Cook et al., 2012).

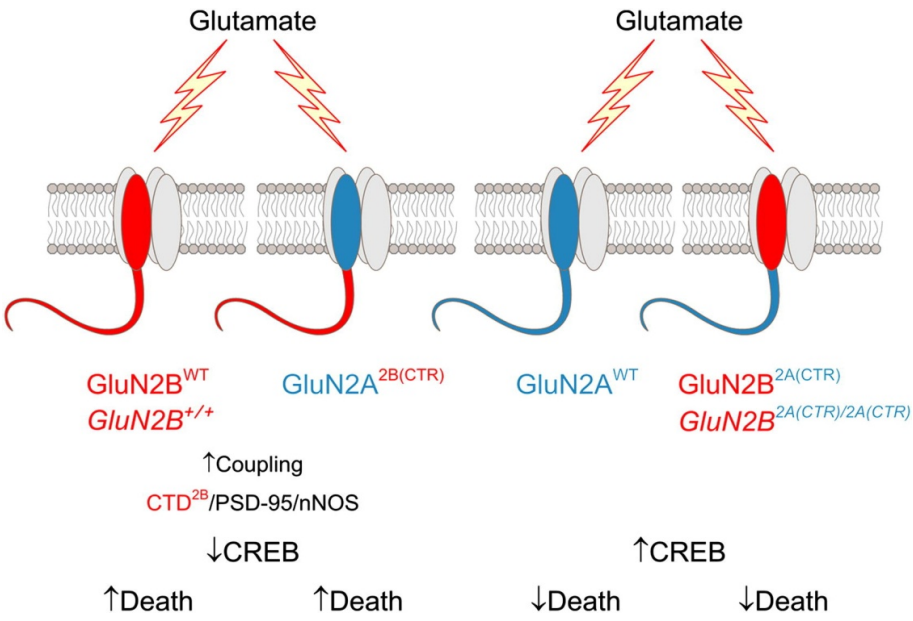


Figure 1.5. Differential contribution of GluN2A and GluN2B subunits to excitotoxic neuronal death. The C-terminal domain of GluN2B subunits is more lethal than that of GluN2A. The extent of cell death upon excitotoxic stimulation depends in part on the C-terminal domain of NMDAR subunits. Swapping the C-terminal domain of GluN2A for GluN2B produces more cell death. Conversely, swapping the C-terminal from GluN2B for GluN2A reduces cell death, due in part to increased phosphorylation of CREB. The C-terminal domain of GluN2B displays stronger coupling to the PSD95/nNOS pathway, which suppresses CREB activation. This indicates that the GluN2B subunit, regardless of location at synaptic or extrasynaptic sites, is more lethal than the GluN2A subunit. (Cepeda and Levine, 2012)

NMDAR Membrane Localization

The majority of NMDARs localizes to highly specialized structures called postsynaptic densities (PSDs). However, they can also be targeted to extrasynaptic sites (Brickley et al., 2003; Thomas et al., 2006; Petralia et al., 2010), and also perisynaptic (Zhang and Diamond, 2009) and presynaptic (Bidoret et al., 2009) sites. The PSDs possess a complex web of scaffold, adaptor and downstream signaling proteins. Two scaffold proteins, PSD95 and synapse-associated protein 102 (SAP102), are abundant at the PSD strucutures, and contain PSD95/Dlg/ZO-1 (PDZ) domains that have the ability to interact with the NMDARs and control their trafficking and synaptic delivery (Kim and Sheng, 2004). Receptors enter and exit the synapse via lateral diffusion mechanisms. In fact, NMDARs have been shown to undergo rapid synaptic exchange (Tovar and Westbrook, 2002) and to diffuse between synaptic and

extrasynaptic sites (Groc et al., 2004). Extrasynaptic NMDARs may function as a highly mobile pool of receptors that can rapidly diffuse to synaptic sites upon mechanisms of activity-induced plasticity. Interestingly, PKC activation seems to regulate both lateral mobility and synapse dispersal of NMDARs (Fong et al., 2002; Groc et al., 2004), which may imply the existence of membrane microdomains that could also be associated with endocytic zones (Blanpied et al., 2002). Furthermore, subunit composition may also contribute to the control of NMDAR surface targeting.

Role of NMDAR Localization in Excitotoxic Mechanisms

Localization of NMDARs at the membrane is thought to have distinct impact on neuronal fate, upon excitotoxic stimulation. Indeed, synaptic NMDARs are considered to induce neuroprotection, whilst NMDARs located extrasynaptically are thought to promote pro-death signaling (Figure 1.6) (reviewed in Hardingham and Bading, 2010).

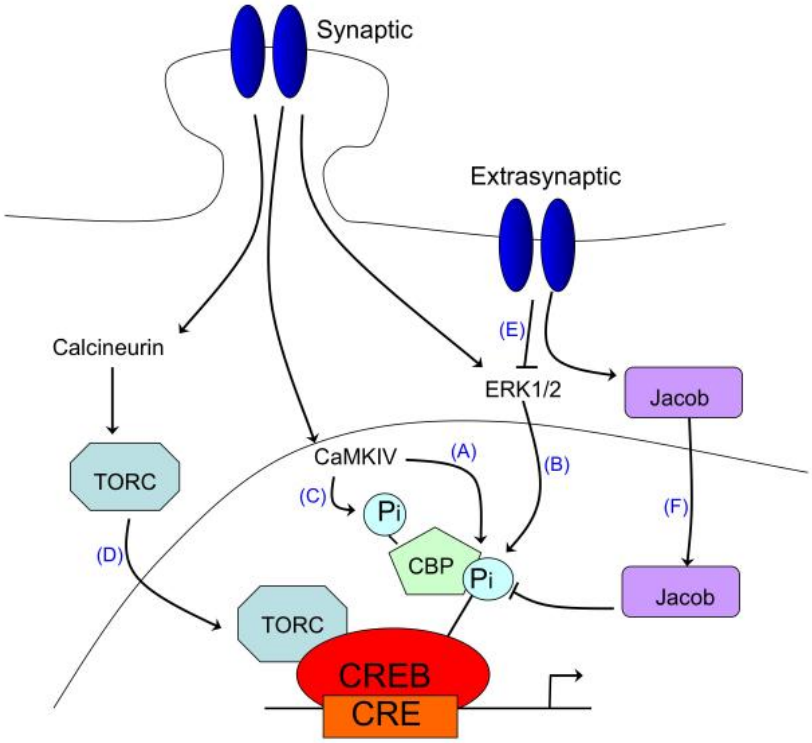


Figure 1.6. Differential contribution of synaptic and extrasynaptic NMDAR activation to excitotoxic neuronal death. Synaptic NMDARs activate CREB-dependent transcription. CREB is phosphorylated at Ser133 by CaMK (A) or ERK1/2 (B), in order to recruit its coactivator CREB binding protein (CBP). CBP is phosphorylated at Ser301 by the nuclear Ca^{2+}-dependent CaMKIV (C). Synaptic NMDAR-induced Ca^{2+} signals promote transducer of regulated CREB activity (TORC) import into the nucleus via

calcineurin-dependent dephosphorylation (D). In contrast to these CREB-activating signals of synaptic NMDARs, extrasynaptic NMDARs suppress CREB activity through inactivation of the Ras-ERK1/2 pathway (E) and the nuclear translocation of Jacob, which promotes CREB dephosphorylation (F). (Adapted from Hardingham and Bading, 2010)

Synaptic NMDAR signaling is thought to induce neuronal survival via several mechanisms. One of the most prominent pathways of neuroprotection occurs through nuclear Ca^{2+} signaling, which induces gene expression (Zhang et al., 2007; Zhang et al., 2009b; Hardingham and Bading, 2010). Among these is the protein CaMKIV, which regulates the activity of the prosurvival transcription factor cAMP response element-binding protein (CREB) (Chrivia et al., 1993; Enslen et al., 1994; Matthews et al., 1994; Hardingham et al., 1997; Mayr and Montminy, 2001; Lonze et al., 2002). Also, the activity of the mitogen-activated protein kinase (MAPK) extracellular-signal regulating kinase 1/2 (ERK1/2), which leads to the activation of CREB, is stimulated by synaptic NMDAR signaling (Chandler et al., 2001; Papadia et al., 2005; Hetman and Kharebava, 2006). Increased CREB transcriptional activity induces the expression of pro-survival genes (for example *Atf3*, *Gadd45b*, *Serpinb2*) (Zhang et al., 2007; Zhang et al., 2009b; Zhang et al., 2011). CREB also stimulates *Bdnf* expression, a known prosurvival neurotrophin (Thoenen et al., 1987; Hansen et al., 2004; Almeida et al., 2005). Concomitantly with induction of prosurvival signaling, synaptic NMDARs also promote neuroprotection by suppressing prodeath signaling. These receptors trigger signaling that suppresses the expression of pro-apoptotic proteins, like Puma (Lau and Bading, 2009; Leveille et al., 2010) and inhibit the activity of pro-death transcription factors such as forkhead O (FOXO) proteins (Lehtinen et al., 2006; Salih and Brunet, 2008; Dick and Bading, 2010). Additionally, synaptic NMDAR activity also seems to improve the anti-oxidant defenses, thereby further contributing to neuronal survival (Papadia et al., 2008).

Activation of extrasynaptic NMDARs antagonizes the effect of synaptic receptors by inducing CREB shut-off. This occurs via coupling the extrasynaptic NMDARs with a dominant CREB-dephosphorylating pathway, performed by the protein Jacob (Hardingham and Bading, 2002; Hardingham et al., 2002; Dieterich et al., 2008; Karpova et al., 2013). Additionally, upon activation of extrasynaptic NMDARs, ERK1/2 activity is rapidly decreased (Bading and Greenberg, 1991; Chandler et al., 2001; Kim et al., 2005; Ivanov et al., 2006; Leveille et al., 2008). Also, extrasynaptic NMDAR signaling leads to FOXO transcription factor activation, contrary to the effect of synaptic

NMDARs (Dick and Bading, 2010). Extrasynaptic NMDARs also induce the activation of other deleterious proteins, such as calpains, as confirmed by cleavage of calpain-specific substrates (Xu et al., 2009). For example, NCX3, which is responsible for removal of Ca^{2+} from the cytoplasm, upon cleavage by calpains accelerates the disruption of Ca^{2+} homeostasis which is highly damaging to neurons (Bano et al., 2005; Araujo et al., 2007). Calpains also promote cleavage of striatal-enriched tyrosine phosphatase (STEP), which leads to disinhibition of the pro-death p38 MAPK, allowing this kinase to be activated and phosphorylate its substrates (Kawasaki et al., 1997; Xu et al., 2009). Overall, extrasynaptic NMDARs contribute to neuronal death by concomitantly suppressing pro-survival pathways and activating deleterious signaling cascades. Despite the plethora of evidence showing a pro-survival role of synaptic NMDARs, these have also been shown to be capable of inducing excitotoxic neuronal death (Papouin et al., 2012).

NMDAR Interactors

Some of the best studied interactors of NMDAR subunits are proteins of the membrane associated guanylate kinase (MAGUK) family (Gardoni et al., 2009). These proteins function as NMDAR scaffolds and as organizers of the PSD, coupling signaling complexes to receptor activity. The members of the MAGUK family, which comprises PSD93, PSD95, SAP97 and SAP102, interact with GluN1 and GluN2 subunits (Zheng et al., 2011). Interestingly, GluN2A was shown to have higher affinity for PSD95 while GluN2B interacts preferentially with SAP102, which may impact on receptor localization. Additionally, the complex between NMDARs and the scaffolds may occur early in the secretory pathway, for example in the ER (Sans et al., 2005). Notably, the interaction of GluN2B-containing NMDARs with PDZ-containing proteins seems to be essential for synaptic localization (Prybylowski et al., 2005; Yi et al., 2007) but not for their targeting to extrasynaptic sites (Sans et al., 2005). This protein family is responsible for coupling NMDARs to downstream signaling pathways. One of the most remarkable examples is the coupling to nNOS (Sattler et al., 1999), which has been shown to have detrimental effects upon excitotoxic injury (Aarts et al., 2002; Cao et al., 2005; Zhou et al., 2010; Cook et al., 2012).

NMDAR Internalization

Contrary to AMPARs, which are rapidly internalized in a synaptic activity-regulated manner (Ehlers, 2000; Lin et al., 2000), NMDARs are considered to be relatively stable in the synapse, even during synaptic plasticity phenomena, in mature neurons (Roche et al., 2001). There are, however, certain stimuli that lead to NMDAR internalization (Snyder et al., 2001; Nong et al., 2003). The GluN2B subunit has two C-terminal domains that regulate the synaptic localization of NMDARs: the PDZ-binding domain and the clathrin adaptor protein 2 (AP2)-binding motif, corresponding to the amino acid sequence YEKL. This is a consensus internalization motif, through which GluN2B interacts with the μ2 subunit of AP2, thus linking the receptor to clathrin-coated pits. GluN2A subunits also possess an AP2 binding motif (LL) on its C-terminus (Lavezzari et al., 2004). Interestingly, there is preferential interaction of GluN2B subunits with AP2, relatively to GluN2A. This fact may justify the decrease in the internalization rate of NMDARs in mature neurons, since GluN2A subunit expression increases with development (Groc and Choquet, 2006). Notably, subunit composition influences the sorting of the endosomes: GluN2B-containing receptors are associated with recycling endosomes and are probably later reinserted to the plasma membrane, while GluN2A-containing receptors are sorted to late endosomes and degraded (Lavezzari et al., 2004; Scott et al., 2004). Both subunits possess a membrane-proximal endocytic motif that targets the receptors for late endosomes (Scott et al., 2004).

Cerebral ischemia induces a plethora of mechanisms that contribute to neuronal demise, beyond excitotoxicity. Thus, we proposed to study an additional mechanism related to induction of a novel programmed cell death process, necroptosis, that is mediated by death receptor (DR) signaling.

Death Receptor Signaling and Cell Fate

Tumor necrosis factor α (TNFα) is a pleiotropic cytokine involved in the initiation and regulation of proinflammatory and immune responses (Gruen and Weissman, 1997; Makhatadze, 1998). It induces the expression of adhesion molecules on the vascular endothelium allowing the recruitment of leukocytes and immune cells to areas

of tissue damage and infection (Gamble et al., 1985; Barbara et al., 1996). This cytokine is produced as a membrane-bound precursor molecule of 26 kDa that is processed by the TNFα converting enzyme (TACE) to produce a 17 kDa active cytokine and binds TNF receptors (TNFRs), which are constitutively expressed in neurons and glia (Benveniste and Benos, 1995). The TNFα receptors include TNFR1 and TNFR2. The latter does not possess a death domain (DD), thus TNFR1 is thought to play a major role in TNFα-induced intracellular signaling.

In the brain, TNFα is produced in its majority by activated microglia and astrocytes, along with other proinflammatory mediators such as the cytokines interleukin-1 (IL-1) and IL-6 and the chemokine interferon gamma (IFNγ), to promote neuroinflammation. Secretion of these cytokines by glial cells induces their autocrine production, further enhancing the expression of TNFα and astrogliosis (Rao et al., 2012). To a lesser extent, TNFα has also been shown to be secreted by neurons (Breder et al., 1993). Increased levels of TNFα have been shown to occur in several neurological disorders, such as AD (Rubio-Perez and Morillas-Ruiz, 2012), multiple sclerosis (MS) (Rieckmann et al., 1995) and brain ischemia (Tuttolomondo et al., 2012).

In a context of *in vitro* global cerebral ischemia, the neurotoxic effect of TNFα has been demonstrated. Indeed, cortical cultures of $TNF^{-/-}$ animals display enhanced protection against the OGD challenge. Additionally, treating wild-type (WT) cultures with neutralizing antibodies against TNF has similar prosurvival effects. This neuroprotection has also been shown in the mouse model of focal ischemia, MCAO (Martin-Villalba et al., 2001). Also, if TNFα is exogenously added after the insult, it exacerbates OGD-mediated injury (Wilde et al., 2000). OGD induces increased TACE activity and expression, leading to increased TNFα secretion, which seems to contribute to the observed inducible NOS (iNOS) activity following OGD (Hurtado et al., 2001). The increased TACE expression seems to occur in microglia and astrocytes, both following OGD and glutamate exposure (Hurtado et al., 2001; Hurtado et al., 2002). Additionally, OGD induces mRNA and protein expression of TNFα and TNFR1 in cortical neurons and microglia, but not in astrocytes (Badiola et al., 2009). Interestingly, OGD has been shown to down-regulate miR-181c, which controls microglia-induced neuronal apoptosis by reducing TNFα expression (Zhang et al., 2012). Notably, if TNFα is present prior to the OGD challenge, it exerts a pro-survival effect, via nuclear factor kappa B (NF-κB) transcriptional activity (Wilde et al., 2000; Romera et al., 2004).

DEATH RECEPTORS

Death receptor signaling is rather complex because it may induce a variety of outcomes, concerning cellular fate. Indeed, upon activation due to ligand binding (for example, TNFα – Table I), it may lead to increased NF-κB-mediated gene transcription, which contributes to cell survival and inflammation but, depending on the molecular context of the cell, DRs may also activate mechanisms of programmed cell death. Specifically, DR signaling may activate either the extrinsic pathway of apoptosis, upon assembly of the death inducing signaling complex (DISC), or a recently described type of programmed necrosis, called necroptosis, which is mediated by a signaling complex called necrosome (Weinlich et al., 2011).

The decision between life and death starts upon TNFα stimulation (Figure 1.7), with the recruitment of DD-containing proteins to the vicinity of the TNFR1 – complex I – which includes, among others, the proteins receptor-interacting protein kinase 1 (RIP1), TNFR-associated factor 2 (TRAF2) and TNFR-associated via death domain (TRADD) (Micheau and Tschopp, 2003). Also, cellular inhibitor of apoptosis protein 1 (cIAP1) may be found in this complex, which leads to survival signaling mediated by NF-κB transcriptional activity, since cIAP1 acts as an inhibitor of death ligand (DL)-induced apoptosis (Wang et al., 1998; Yang and Du, 2004; Gaither et al., 2007; Geserick et al., 2009). When NF-κB signaling is impaired, a second complex with high molecular weight is formed – complex II. This complex comprises RIP1, TRADD, TRAF2, Fas-associated protein with death domain (FADD) and procaspase-8 and is dissociated from TNFR1, which is internalized. In these conditions, cells are able to undergo apoptosis, through processing of caspases-8, -2 and -3 (Micheau and Tschopp, 2003). Inhibition of caspases sensitizes some cells, such as L929 cells, to TNF-mediated necrosis, which involves the formation of a third complex, complex IIB or necrosome (Declercq et al., 2009) and reactive oxygen species (ROS) production (Vercammen et al., 1998; Cho et al., 2009).

Table I. Death Receptor Superfamily members

Receptor	Ligand	Features	Interactions
Fas/CD95	FasL	One DD	FADD
TNFR1	TNFα	One DD	TRADD
TNFR2	TNFα	-	TRAF2
DR3/APO-3	APO-3L/TWEAK	One DD	TRADD
DR4/5	APO-2L/TRAIL	One DD	TRADD

The ubiquitination state of RIP1 seems to be determinant for induction of complex II assembly. This post-translational modification of RIP1 is highly regulated by both ubiquitinases and deubiquitinases (DUBs). Indeed, cIAP1 and cIAP2 are able to ubiquitinate RIP1 and upon inhibition of these enzymes the recruitment of RIP1 to complex II is increased (Geserick et al., 2009). The DUB Cezanne leads to suppression of NF-κB signaling in response to TNFα activation by removing Lys63 polyubiquitin chains from RIP1, in complex I. This contributes to increased stability of the inhibitor of kappa B (IκB) complex, which retains NF-κB on the cytoplasmatic compartment (Enesa et al., 2008). Inhibition of both cIAP1 and transforming growth factor-β activated kinase-1 (TAK1) induces the formation of the necrosome and subsequent ROS production, via increased recruitment of RIP3 and RIP1 kinase activity (Vanlangenakker et al., 2011b). Interestingly, TAK1 seems to have a function as an adaptor molecule, independently of its kinase activity, preventing premature dissociation of RIP1 from complex I and induction of necroptosis (Arslan and Scheidereit, 2011). Although RIP1 is generally thought to be necessary for canonical NF-κB activation (Ting et al., 1996; Kelliher et al., 1998; Ea et al., 2006; Li et al., 2006), there evidence shows that it may not be essential. Indeed, in $RIP1^{-/-}$ mouse embryonic fibroblasts (MEFs), the cells are still able to induce NF-κB transcriptional activity, through increased degradation of IκBα (Wong et al., 2010). Interestingly, HeLa cells treated with etoposide to induce DNA damage were shown to induce biphasic NF-κB activation, with differential regulation. RIP1 kinase activity was shown to be exclusively necessary for the second phase of NF-κB activity. Furthermore, this type of insult was shown to induce the recruitment of FADD and caspase-8 to a RIP1-NF-κB essential modulator (NEMO) complex, thereby initiating apoptotic signaling, which is prevented by knock-down (Tia et al.) of either RIP1 or caspase-8 (Biton and Ashkenazi, 2011).

The association between RIP1 and caspase-8 in complex II seems to be highly dependent on FADD availability, since treatment of cells with a dominant negative form of FADD (FADD-DN) renders cells resistant to TNF-induced apoptosis and impairs this interaction. This effect can be rescued by expression of WT FADD (Micheau and Tschopp, 2003). The protein FADD can, in fact, have a deep impact on the phenotype of cell death being activated following TNFR1 signaling. Different domains of this protein have the ability to induce caspase-dependent apoptosis (death effector domain – DED) or necrosis (DD), in a caspase-independent manner. This seems to be regulated by their ability to selectively interact with caspase-8 or RIP-1, respectively (Vanden Berghe et al., 2004).

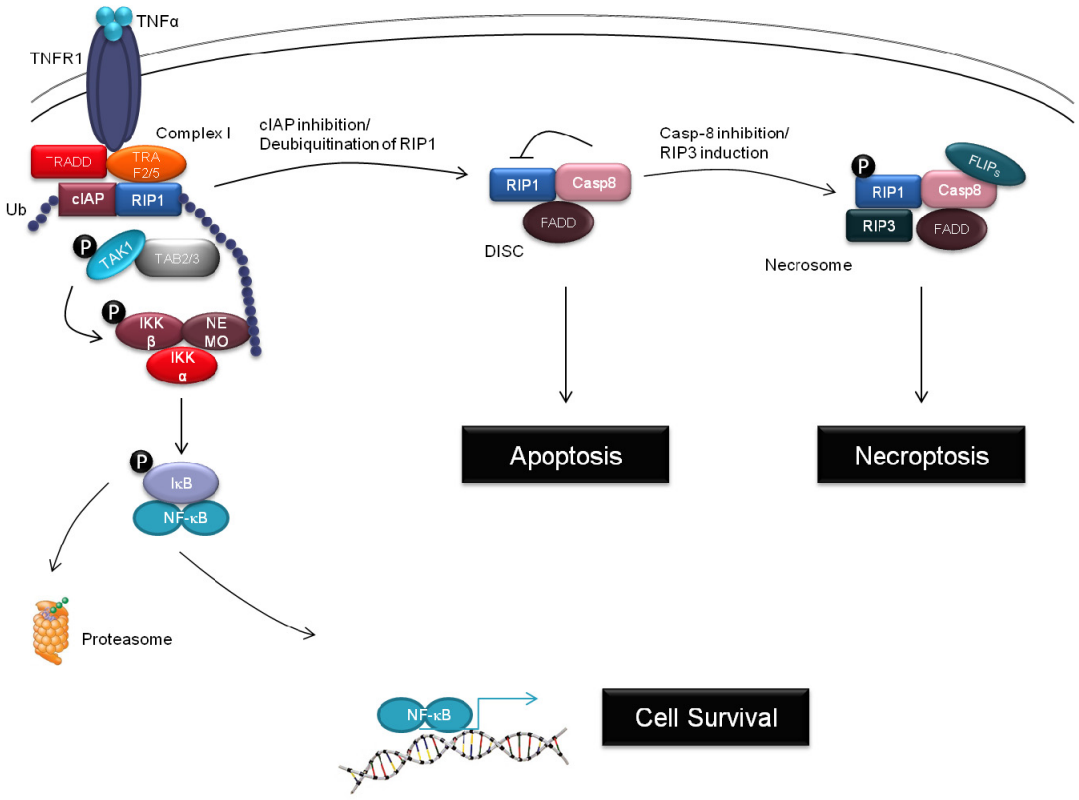


Figure 1.7. DR signaling complexes. Upon DL binding, the DR recruits a complex of DD-containing proteins to its vicinity (complex I). In this complex, RIP1 is ubiquitinated by cIAPs, initiating signaling to NF-κB activation. This contributes to cell survival via transcription of survival genes. Upon deubiquitination of RIP1, the complex dissociates from the receptor and recruits FADD and procaspase-8, forming a complex called DISC. Caspase-8 auto-activates, cleaves RIP1 inhibiting necroptosis, and initiates apoptosis. In conditions of low caspase-8 activity, RIP3 is recruited to interact with RIP1, assembling the necrosome, and together they activate necroptosis.

Apoptosis and necroptosis are complementary cell death pathways, as demonstrated by occlusion of the lethality observed in caspase-$8^{-/-}$ mice when RIP3 is concomitantly depleted, in caspase$8^{-/-}$/RIP$3^{-/-}$ double knock-out (KO) mice (Kaiser et al., 2011; Oberst et al., 2011), which emphasizes the role of caspase-8 as a negative regulator of necroptosis. This regulation is achieved by the formation of catalytically active dimers of caspase-8 and FLICE-like inhibitory protein long ($FLIP_L$), which inhibit the assembly of the necrosome, without inducing apoptosis (Micheau et al., 2002; Oberst et al., 2011). Interestingly, RIP3 overexpression is not lethal *per se*, but sensitizes MEFs to TNFα treatment, in a RIP1 and caspase-8 independent manner (Moujalled et al., 2013).

Although TNFα and its receptor TNFR1 are the most commonly studied in necroptosis research, other DLs and their respective DRs might have a role in induction of cell death signaling (reviewed in Han et al., 2011). Indeed, TNF-related weak inducer of apoptosis (TWEAK), which binds DR3, has been shown to induce the formation of a DISC comprising RIP1, FADD and caspase-8, but lacking TRADD, and to induce apoptosis. Interestingly, this DL does not seem to induce activation of necroptosis (Ikner and Ashkenazi, 2011). Also, Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) were shown to induce a form of caspase-independent necrotic cell death that is dependent on RIP1 kinase activity (Holler et al., 2000). The receptor cluster of differentiation 95 (CD95) is also known to induce apoptotic cell death in T cells. Upon caspase inhibition, however, the type of cell death is shifted to necrosis and proinflammatory signaling via ERK and p38. This receptor was also shown to induce NF-κB activity, which lead to increased cytokine production (Scheller et al., 2002). Toll-like receptor 3 (TLR3) signaling has also been shown to activate necroptotic mechanisms of cell death (Kalai et al., 2002).

Survival Signaling

Upon TNFα binding to TNFR1, complex I is recruited to the receptor (Figure 1.8) and RIP1 is ubiquitinated by cIAPs (Bertrand et al., 2008; Varfolomeev et al., 2008; Bianchi and Meier, 2009). The Lys63 chains linked to RIP1 then recruit TAK1 binding protein 2 (TAB2) and NEMO, with the associated kinases (TAK1 and IκB kinase – IKK, respectively) (Kanayama et al., 2004; Ea et al., 2006; Li et al., 2006; Wu et al., 2006a). Linear Ub chain assembly complex (LUBAC) is also recruited to complex I, in an Ub-dependent manner, and was shown to stabilize this complex (Haas et al., 2009). LUBAC is responsible for ubiquitinating NEMO. Linear ubiquitination of this protein was shown to be necessary for TNFR1-induced NF-κB activity (Tokunaga et al., 2009; Tokunaga et al., 2012; Verhelst et al., 2012). Once in this complex, TAK1 phosphorylates IKKβ. Upon activation, IKKβ phosphorylates IκB, targeting it for degradation. This results in the relief of NF-κB inhibition, which is then able to translocate to the nucleus and initiate gene transcription (Karin and Ben-Neriah, 2000).

Activation of NF-κB signaling has been shown to be counteracted by the action DUBs on RIP1, such as A20 (He and Ting, 2002; Wertz et al., 2004) and cylindromatosis (CYLD) (Wright et al., 2007). The A20 ubiquitin-editing enzyme removes Lys63 polyubiquitin chains from RIP1 and concurrently links Lys48

polyubiquitin chains to this protein, thus targeting it for degradation by the proteasome system. This results in concomitant inhibition of NF-κB survival signaling and inhibition of TNF-induced cell death by either apoptosis or necrosis (He and Ting, 2002; Wertz et al., 2004; Vanlangenakker et al., 2011a). On the other hand, CYLD removes Lys63 chains from RIP1, promoting necroptosis (Welz et al., 2011). This DUB is rapidly cleaved by active caspase-8 (O'Donnell et al., 2011) thus, it is only stabilized and capable of deubiquitinating RIP1 upon insufficient activity of caspase-8, which may sensitize the cells to necroptotic death. Contrary to its ubiquitination state, the kinase activity of RIP1 does not regulate NF-κB activation (Lee et al., 2004; Wong et al., 2010).

Besides the known NF-κB signaling regulation, activation of TNFRs also leads to activation of MAPKs. The kinase RIP1 was shown to be necessary for TNFα-induced MAPK activation (ERK, p38 and c-Jun N-terminal kinase – JNK), as observed by reduction of MAPK phosphorylation in RIP1$^{-/-}$ MEFs. The kinase activity of RIP1, however, seems to be only required for ERK activation (Devin et al., 2003; Lee et al., 2003). JNK is thought to contribute to the build-up of oxidative stress, promoting mitochondrial dysfunction, downstream of RIP1 and TRAF2, and leading to necrotic cell death (Davis, 2000; Shen et al., 2004; Xu et al., 2006).

Apoptosis

Apoptosis is generally described as a type of programmed cell death, due to its reliance on specific and sequential cellular mechanisms, in order to be activated. This type of cell death induces several cytoarquitectural alterations, including membrane blebbing, with formation of apoptotic bodies, chromatin condensation, DNA laddering and fragmentation of the nucleus, organelle fragmentation and release of protein content from the mitochondria to the cytoplasm. After cellular deconstruction, the debris are removed by phagocytes, avoiding activation of inflammatory responses and damage to the neighboring cells (Taylor et al., 2008).

There are two major pathways of apoptosis: the extrinsic (or DR pathway) and the intrinsic (or mitochondrial pathway). These pathways are thought to cross-talk and influence one another (Igney and Krammer, 2002) (Figure 1.9).

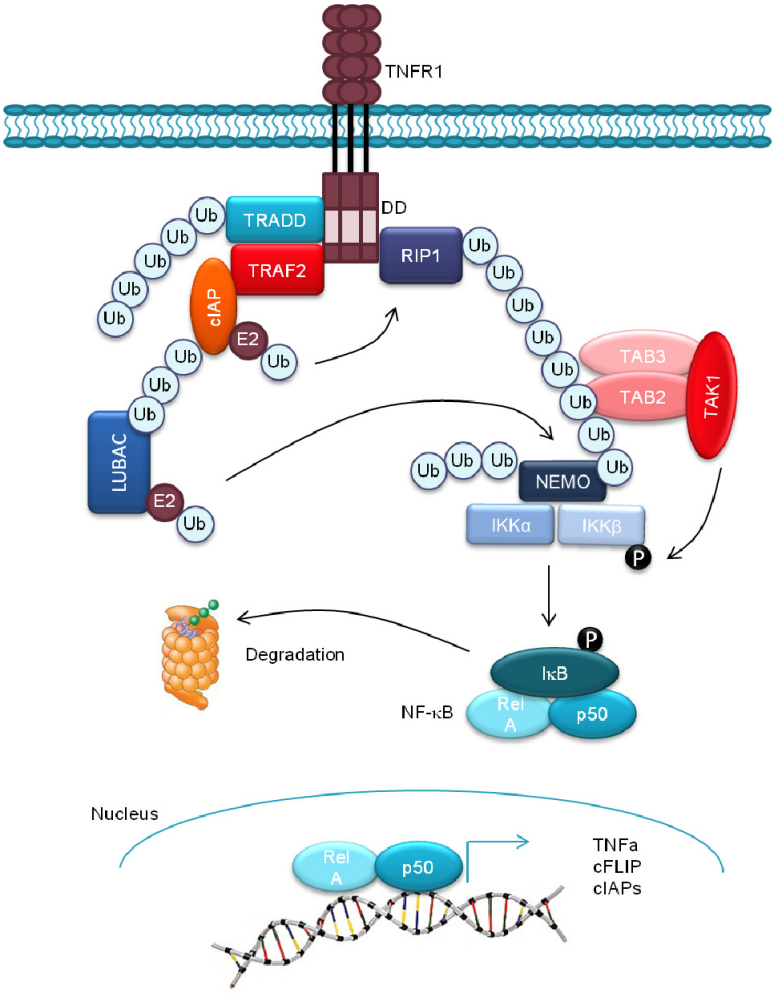


Figure 1.8. Assembly of DR-associated complex I. Upon activation, TRADD, TRAF2 and RIP1 are recruited to interact with the TNFR1, through their DDs. cIAPs ubiquitinate RIP1 and TRADD and RIP1-bound polyubiquitin chains recruit TAB2/3 and NEMO. TAK1 becomes active and phosphorylates IKKβ, which becomes active. This kinase then phosphorylates IκB, which relieves the inhibition exerted on NF-κB dimmers. LUBAC is also recruited to this complex in an Ub-dependent manner and it stabilizes the complex by ubiquitinating NEMO. Upon relief of the inhibition, NF-κB dimmers translocate to the nucleus and initiate gene transcription, promoting cell survival and inflammation.

The extrinsic pathway of apoptosis is initiated by DR signaling. Under appropriate conditions, complex II or DISC is assembled and auto-catalytic activation of caspase-8 occurs (Kischkel et al., 1995). The activation of caspase-8 can be inhibited by cFLIP isoforms ($FLIP_L$, $FLIP_S$ and $FLIP_R$) that bind procaspase-8 keeping it in an inactive state (Kataoka et al., 1998; Scaffidi et al., 1999). This process is regulated by the activity of complex I. Upon NF-κB activation via complex I, the transcription factor

induces, among other antiapoptotic genes, the expression of $FLIP_L$, which then exerts a negative regulatory effect on complex II, through inhibition of caspase-8. However, if complex I displays limited activity, then the expression of $FLIP_L$ will be lower and complex II will have the ability to initiate apoptotic signaling (Micheau and Tschopp, 2003). The $FLIP_L$ protein possesses caspase-like domains, but has no catalytic activity (Tschopp et al., 1998), thus it interacts with procaspase-8, allowing its allosteric activation and partial processing. This event inhibits induction of apoptosis due to caspase-8 limited activity, but allows this enzyme to process local substrates, like RIP1 (Krueger et al., 2001; Micheau et al., 2002; Kavuri et al., 2011). Therefore it facilitates concomitant inhibition of apoptosis and necroptosis (Oberst et al., 2011). The $FLIP_S$ isoform, on the other hand, inhibits caspase-8 auto-activation (Krueger et al., 2001; Golks et al., 2005), thereby limiting the cleavage of RIP1, which is then capable of initiating necroptosis signaling. In conditions where caspase-8 is able to auto-activate, it triggers the execution phase of apoptosis. The extrinsic pathway of apoptosis cross-talks with the intrinsic pathway via caspase-8 mediated truncation of BH3 interacting-domain death agonist (Bidoret et al.). This proapoptotic protein is then able to translocate to the mitochondria, activate the proapoptotic proteins Bax/Bak and promote the formation of outer membrane pores and the release of apoptotic mitochondrial proteins to the cytoplasm (Li et al., 1998a; Esposti, 2002; Kaufmann et al., 2012).

The intrinsic pathway of apoptosis is characterized by mitochondrial dysfunction, resulting in the opening of the mitochondrial pores. This induces loss of mitochondrial membrane potential and release of proapoptotic proteins to the cytoplasm (Saelens et al., 2004). A first group of proteins to be released includes cytochrome c, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) and high temperature requirement protein A2 (HtrA2/Omi) (Du et al., 2000; van Loo et al., 2002; Garrido et al., 2006). Once in the cytoplasm, cytochrome c interacts with apoptotic peptidase activating factor 1 (Apaf-1) and procaspase-9, forming the so-called apoptosome and initiating the caspase-dependent mitochondrial pathway (Chinnaiyan, 1999; Hill et al., 2004). At a later stage, a second group of proteins is released from the mitochondria, after the cell has committed to the death program. This includes apoptosis-inducing factor (AIF) and endonuclease G, which translocate to the nucleus to induce DNA fragmentation and nuclear condensation (Joza et al., 2001; Li et al., 2001). In the cytoplasm, the caspase activated DNase (CAD) is kept in an inactive state by its inhibitor, ICAD. Upon caspase-3 cleavage of ICAD, the active endonuclease is

released and further exacerbates chromatin condensation (Enari et al., 1998; Sakahira et al., 1998).

The regulation of the mitochondrial pathway of apoptosis is mediated by a family of proteins, the B-cell lymphoma-2 (Bcl-2) family, which includes both pro-apoptotic (for example, Bax, Bak, Bim, BID, Bad) and anti-apoptotic members (including Bcl-2, Bcl-X_L, Bcl-x, BAG) (Cory and Adams, 2002). Several other pro- and anti-apoptotic proteins, as well as additional signaling pathways, like MAPK signaling cascades, participate in apoptotic mechanisms, contributing to a very complex program to induce cellular demise.

These two pathways of apoptosis converge to the execution phase, which is performed by active caspases. The executioner caspases (caspase-3, -6 and -7) are activated by the initiator caspases (caspase-8, -9 and -10) by cleavage of the procaspase, and exert their effects via proteolysis of a plethora of substrates. Among these are poly-ADP ribose polymerase (PARP), α-fodrin, endonucleases, ICAD and gelsolin (Slee et al., 2001). The latter is cleaved by caspase-3 causing disruption of the cytoskeleton (Kothakota et al., 1997), which contributes to the cellular demolition characteristic of apoptosis (Taylor et al., 2008).

The final stage of apoptosis is the phagocytic removal of cellular debris. This process is preceded by externalization of phosphatidylserine on the cell surface, which facilitates the recognition of the cells to be phagocytosed, without activation of inflammatory mechanisms (Fadok et al., 2001). This feature, however, is not exclusive of apoptosis (Krysko et al., 2004).

Global ischemic insults have been shown to induce apoptotic neuronal death in the CA1 region of the hippocampus, where the cells display shrunk and condensed nuclei, along with other features related to this type of cell demise (Ozawa et al., 1999). Global ischemia also induces translocation of the pro-apoptotic protein Bad, to the mitochondria, where it assembles with other pro-apoptotic proteins, such as Bcl-X_L. The latter is cleaved to produce a pro-death fragment that promotes the dissipation of mitochondrial membrane potential. This then leads to release of proapoptotic proteins from the mitochondria to the cytosol, such as cytochrome c and Smac/DIABLO. These proteins promote the formation of the apoptosome, which induces activation of caspases, namely the executioner caspase-3. The activity of this caspase is increased after global ischemia, in a region-specific manner, since its activity is observed in CA1 neurons, but not in the CA3/dentate gyrus (DG) area. Caspase-3 then promotes DNA fragmentation and subsequent neuronal death in CA1 neurons (Miyawaki et al., 2008).

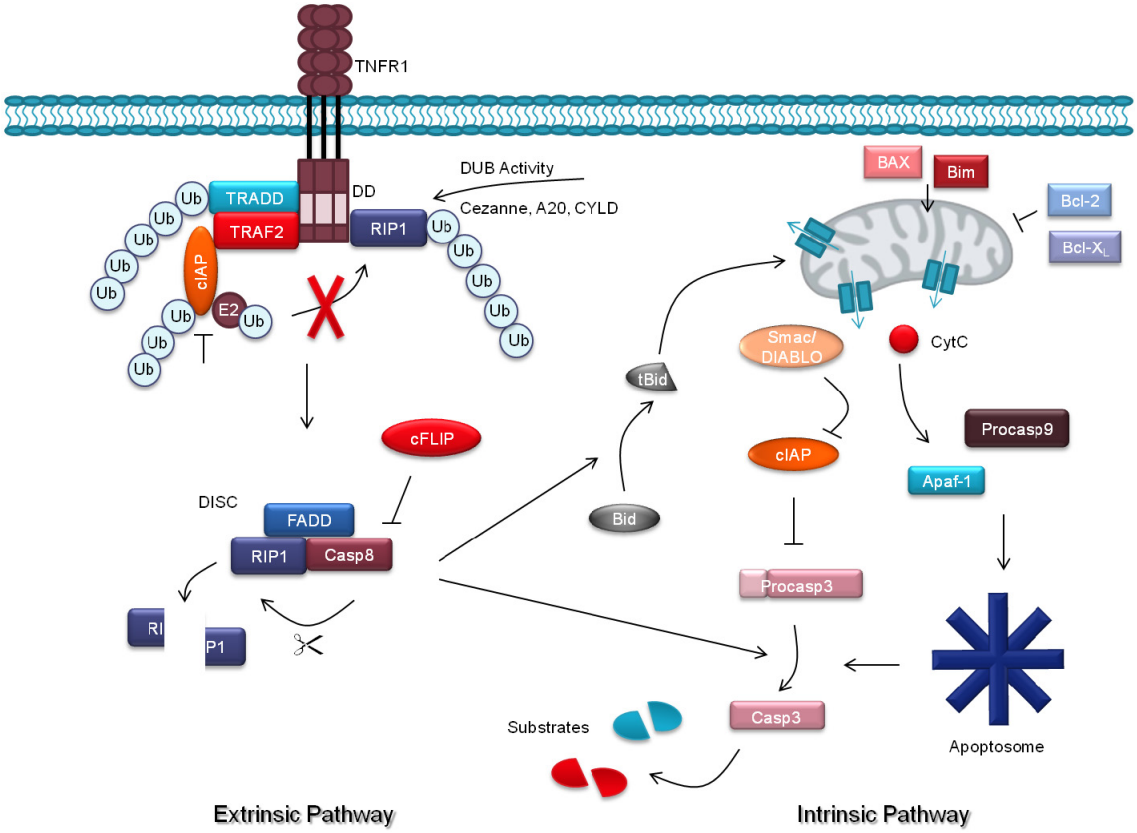


Figure 1.9. Apoptotic Pathways. Apoptosis is elicited in cells via two distinct pathways that cross-talk. Extrinsic pathway: Upon deubiquitination of RIP1, complex I dissociates from TNFR1 and recruits FADD and procaspase8. In the presence of low levels of cFLIP isoforms, caspase8 is able to autoactivate and cleave RIP1, thereby inhibiting necroptosis. Caspase8 truncates BID and processes caspase3, which contributes to the intrinsic pathway of apoptosis. Intrinsic pathway: Apoptotic stimulation of cells leads to mitochondrial dysfunction, with formation of membrane pores. This contributes to the release of pro-apoptotic mitochondrial proteins such as cytochrome C and Smac/DIABLO. This process is regulated by members of the Bcl-2 family, which includes a variety of pro- and anti-apoptotic proteins. In the cytoplasm, cytochrome C interacts with Apaf-1 and procaspase9, assembling the apoptosome. Caspase9 becomes catalytically active and caspase3, a major executioner caspase, is processed, becoming activated. This enzyme exerts its effects by cleaving a variety of intracellular substrates, contributing to apoptotic cell death.

Accordingly, the OGD challenge also induces apoptosis, as demonstrated by typical morphological alterations, as well as activation of specific molecular pathways. Morphologically, there are evidences of DNA laddering and chromatin condensation (Kalda and Zharkovsky, 1999). The OGD challenge also induces depolarization of the

mitochondria, as measured by dissipation of mitochondrial membrane potential (He et al., 2009; Liu et al., 2011), which occurs during apoptotic cell death. Upon induction of OGD in cerebrocortical cultures, both caspases and calpains become activated. This has been shown through the increased formation of spectrin cleavage products of 120 kDa and 145 kDa, which are specific for caspases and calpains, respectively, and also by neuroprotection afforded by caspase (Nath et al., 1998) and calpain inhibitors against OGD-induced death (Newcomb-Fernandez et al., 2001).

The OGD challenge appears to also activate the extrinsic apoptotic pathway. Treatment with an anti-TNFα antibody reduced the number of apoptotic neurons, in cortical cultures submitted to OGD. Additionally, OGD enhances caspase-8 activation mediated by TNFR1 and subsequent caspase-3 activation (Badiola et al., 2009). The OGD challenge also induces cleavage of the pro-apoptotic protein BID, a caspase-8 substrate, before the onset of neuronal death. Furthermore, the cell death elicited by OGD is reduced when BID is inhibited (Culmsee et al., 2005) and in $BID^{-/-}$ cortical neurons, compared to WT neurons. These effects are also observed *in vivo*, using the focal ischemia model MCAO, where $BID^{-/-}$ animals display reduced infarct volumes as well as reduced cytochrome c release from the mitochondria (Plesnila et al., 2001).

On the other hand, members of the anti-apoptotic Bcl-2 family have been shown to have neuroprotective effects against ischemic stimuli. Indeed, cells overexpressing Bcl-2 display increased resistance to the OGD challenge and reduced activation of caspase-3 and cytochrome c release from the mitochondria, as well as diminished loss of mitochondrial membrane potential (Koubi et al., 2005). Moreover, the Bcl-X_L family member was shown to reduce nuclear translocation of AIF as well as the extent of cell death induced by OGD (Cao et al., 2003).

Futhermore, the anti-apoptotic protein Apaf-1 interacting protein (AIP), which acts by preventing apoptosome formation, has been demonstrated to be up-regulated in the regions of the hippocampus that are resistant to OGD-induced damage, but not in the CA1 area. Furthermore, overexpressing AIP in CA1 neurons significantly reduces OGD- or global ischemia-induced neuronal death and caspase-3 activation (Cao et al., 2004a).

Necroptosis

Caspase-independent cell death that occurs as a consequence of DR-mediated signaling is considered to be a type of regulated necrotic cell death because it displays necrotic features, such as plasma membrane damage, swollen mitochondria and slow mitochondrial membrane depolarization, subsequent to nuclear and cytoplasmatic

morphological changes. Also, mitochondrial apoptotic markers like cytochrome C release or caspase-3-dependent cleavage of PARP, as well as chromatin condensation or oligonucleosomal DNA degradation, typical features of apoptosis, are not observed in this type of cell death (Holler et al., 2000; He et al., 2009; Vandenabeele et al., 2010), hence its classification as programmed necrosis (necroptosis).

Necroptotic stimulation, such as a combination of Smac mimetics (which contribute to cIAP1/2 degradation) and caspase inhibitors, such as N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me)-fluoromethyl ketone (zVAD.fmk), leads to a necrotic phenotype, in a DR-dependent manner, as demonstrated by KD of TNFR1 or treatment with a TNFα neutralizing antibody (He et al., 2009). RIP1 was shown to regulate autocrine TNFα production, in a NF-κB-independent manner. In certain contexts, such as caspase inhibition (with zVAD.fmk), cIAP1/2 depletion (with Smac mimetics) or absence of TRAF2, RIP1 associates with the protein E3 ubiquitin-protein ligase UBR5 (EDD) and together they activate the JNK signaling pathway. JNK then phosphorylates its substrates activator protein-1 (AP-1) and specificity protein 1 (Sp-1), which become transcriptionally active, inducing TNFα expression and subsequent inflammation and cell death by necrosis (Christofferson et al., 2012).

In an effort to elucidate the mechanism of necroptosis that is activated in response to DR signaling, three independent groups identified RIP3 as a protein that is specifically associated with necroptotic signaling, using different approaches (Cho et al., 2009; He et al., 2009; Zhang et al., 2009a). The failure of a RIP3-DN to prevent FADD-induced apoptosis (Feng et al., 2006) is in accordance with these reports that relate RIP3 specifically to necroptosis.

Cho and colleagues (2009) performed a siRNA screen in FADD-deficient Jurkat cells, which are known to die by necroptosis in response to TNFα. They identified both RIP1 and RIP3 as crucial mediators of this type of cell death. Interestingly, they also found that RIP3 KD had no effect on TNF- and FasL-induced apoptosis or NF-κB activation. This emphasizes the specific role of RIP3 on the necroptotic mechanism. This protein was shown to be specifically recruited to complex II but, unlike RIP1, was not present in complex I (Cho et al., 2009).

He et al. (2009) adopted a similar strategy. They performed a genome-wide siRNA screen and transfected HT-29 cells with different siRNA pools to assess which of these were protective against necroptotic stimulation. Besides RIP1, which had been previously identified, they found RIP3 to be important for this process. Importantly, this

protein was specifically correlated with necroptosis since it did not interfere with induction of apoptosis. In fact, the ability of different cell lines to undergo necroptosis seems to be correlated with RIP3 expression. Accordingly, cells that express RIP3 have the ability to induce necroptosis (for example Jurkat, U937, L929 and MEFs) while cells with no observable expression of RIP3, are resistant to necroptotic stimulation, including HEK293T, MCF-7 and U2OS. Interestingly, when they expressed RIP3 in "resistant" cells, like T98G cells, they rendered the cells susceptible to necroptosis (He et al., 2009).

Zhang et al. (2009) studied the mechanism of necroptosis in two lines of NIH3T3 cells, N and A cells, which are, respectively, susceptible and resistant to necroptosis. They performed a microarray analysis to detect changes in genes that might be responsible for this differential vulnerability and found that RIP3 was responsible for induction of necroptosis in N cells, while A cells lack expression of this protein. Specifically, they demonstrated that KD of RIP3 abolishes the component of necroptotic death in N cells, whereas expression of RIP3 in A cells renders them susceptible to TNF-induced necroptosis (Zhang et al., 2009a).

The human RIP3 gene has been shown to produce a full length (FL-RIP3) protein and two splice variants – RIP3β and RIP3γ. The FL-RIP3 is widely expressed throughout the organism, including the heart, brain, lung and spleen. While the RIP3γ variant displays comparable mRNA expression to that of the FL-RIP3, in most tissues, the RIP3β variant is expressed at very low levels (Yang et al., 2005). Unlike FL-RIP3 (Yang et al., 2004), both RIP3γ and RIP3β seem to be unable to shuttle between the cytoplasm and the nucleus, which may be related to their inability to induce cell death (Yang et al., 2005). The RIP3 kinase activity is required for nuclear localization of the protein and to the induction of caspase-independent cell death. In fact, RIP3 seems to translocate to the nucleus upon induction of cell death (Feng et al., 2007).

RIP1 and RIP3 where demonstrated to associate through their RIP homotypic interaction motif (RHIM) domains (Sun et al., 2002), forming an amyloid complex upon induction of necroptosis (Li et al., 2012) (Figure 1.10). Additionally, RIP3 was shown to autophosphorylate and phosphorylate RIP1. Phosphorylation of RIP1 seems to act as a cue to shut-off NF-κB signaling, which contributes to cell death (Sun et al., 2002) and it may contribute to the stabilization of the association of RIP1 within complex II (Cho et al., 2009). Also, the kinase activity and the RHIM domains of RIP1/3 are crucial for induction of necroptosis (Cho et al., 2009; He et al., 2009; Zhang et al., 2009a; Li et al., 2012). Mutations in the RHIM domain of RIP3 abolished its ability to induce necroptosis

(He et al., 2009). Necrostatin-1 (Nec-1) a pharmacological inhibitor of necroptosis, was found to inhibit RIP1 kinase activity, by inhibiting, for example, RIP1 autophosphorylation. In fact, four putative autophosphorylation sites were identified (Ser14/15, Ser20, Ser161 and Ser166), all in the N-terminal domain, which is also the kinase domain of RIP1 (Degterev et al., 2008). The pronecrotic complex II, called necrosome, seems to be distinct from the apoptotic one (DISC) since the interaction between RIP1 and RIP3 appears to occur specifically under necrotic-inducing conditions (Cho et al., 2009) (Figure 1.11). Interestingly, RIP1-independent (but RIP3-dependent) necroptosis has been shown to occur upon KD of both RIP1 and caspase-8, evidencing once again the negative regulation exerted by caspase-8 and suggesting that RIP3 may be sufficient to induce necroptosis in certain cellular contexts (Vanlangenakker et al., 2011a). The intermediate domain (ID) of RIP1 seems to be important for induction of necroptosis, as deletion of this domain in the protein shifts the type of cell death from necroptosis to apoptosis, due to increased recruitment of RIP1 and caspase-8 to FADD (Duprez et al., 2012).

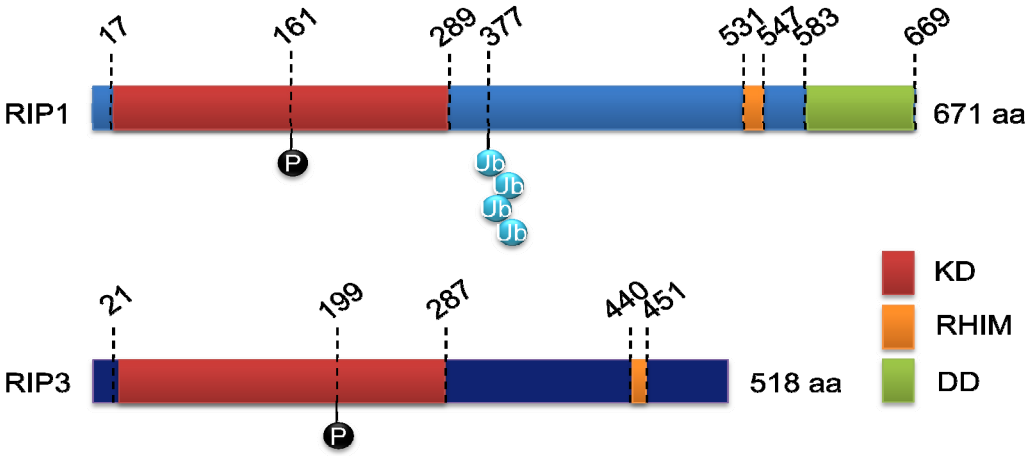


Figure 1.10. Struture of the human RIP1/3 proteins. RIP1 and RIP3 share partial homology in their structure. They both contain a N-terminal kinase domain and a RHIM domain, through which they interact upon necroptosis induction. RIP1 also possesses a C-terminal DD, which allows this protein to interact with other DD-containing proteins (for example, TNFR1) in complex I and II.

This pronecrotic interaction between RIP1 and RIP3 was recently shown to be regulated by the acetylation state of RIP1. Under basal conditions, RIP3 interacts with the deacetylase sirtuin2 (SIRT2). Upon induction of necrosis, the RIP3-SIRT2 complex is recruited to RIP1, thereby facilitating SIRT2-dependent deacetylation of RIP1 on

Lys530. Due to the close proximity of this residue to the RHIM domain of RIP1, which comprises residues 531-547 of RIP1, acetylation on Lys530 possibly disrupts the interaction with RIP3. Upon deacetylation of this residue, RIP1 and RIP3 are able to interact and necroptosis ensues. This evidence is further supported by the fact that SIRT2$^{-/-}$ MEFs are protected from necrosis-inducing insults (Narayan et al., 2012).

Mixed lineage kinase domain-like (MLKL) was shown to be a molecular target of the necrosome (Sun et al., 2012; Zhao et al., 2012). Its role in necroptosis is emphasized by complete abrogation of necroptosis upon MLKL KD or in the presence of necrosulfonamide, a MLKL inhibitor. The mitochondrial protein Ser/Thr-protein phosphatase (PGAM5) is also phosphorylated by RIP3 and associates with the RIP1/3-MLKL complex. The short isoform of PGAM5 (PGAM5S) is proposed to be an effector downstream of the necrosome since its RIP3-mediated phosphorylation is abrogated by necrosulfonamide. PGAM5S then dephosphorylates the mitochondrial protein dynamin related protein 1 (Drp-1), resulting in its activation, which contributes to mitochondrial fragmentation (Wang et al., 2012).

The cellular signaling network involved in the necroptotic process was shown to be regulated by several genes (Hitomi et al., 2008) and requires *de novo* protein synthesis (Yu et al., 2004). Mitochondrial dysfunction, ROS accumulation and ATP depletion have been shown to contribute to the detrimental effect of necroptosis, which is prevented in RIP1$^{-/-}$ cells (Cho et al., 2009; He et al., 2009; Irrinki et al., 2011). The RIP3 kinase promotes mitochondrial ROS generation via a group of metabolism-related enzymes, known to associate with RIP3: phosphorylase-glycogen-liver (PYGL), glutamate-ammonia ligase (GLUL) and glutamate dehydrogenase 1 (GLUD1). These enzymes are involved in mitochondrial metabolism. The protein PYLG is the rate-limiting enzyme in glycogen degradation, while GLUL and GLUD1 are determinant enzymes for oxidative phosphorylation (Zhang et al., 2009a). This seems to contribute to the insertion of the proapoptotic protein BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNip3) in the mitochondria, contributing to mitochondrial damage. Interestingly, BNip3 is induced following TNFα stimulation in alveolar lung cells, but it is not involved in the extrinsic pathway of apoptosis but instead in induction of necroptosis (Kim et al., 2011). Another Bcl-2 family member, Bmf, was also shown to be important for necroptosis induction as KD of this protein inhibits necroptotic cell death (Hitomi et al., 2008). These reports emphasize the complex interplay and common players found between these two distinct programs of cell death: apoptosis and necroptosis.

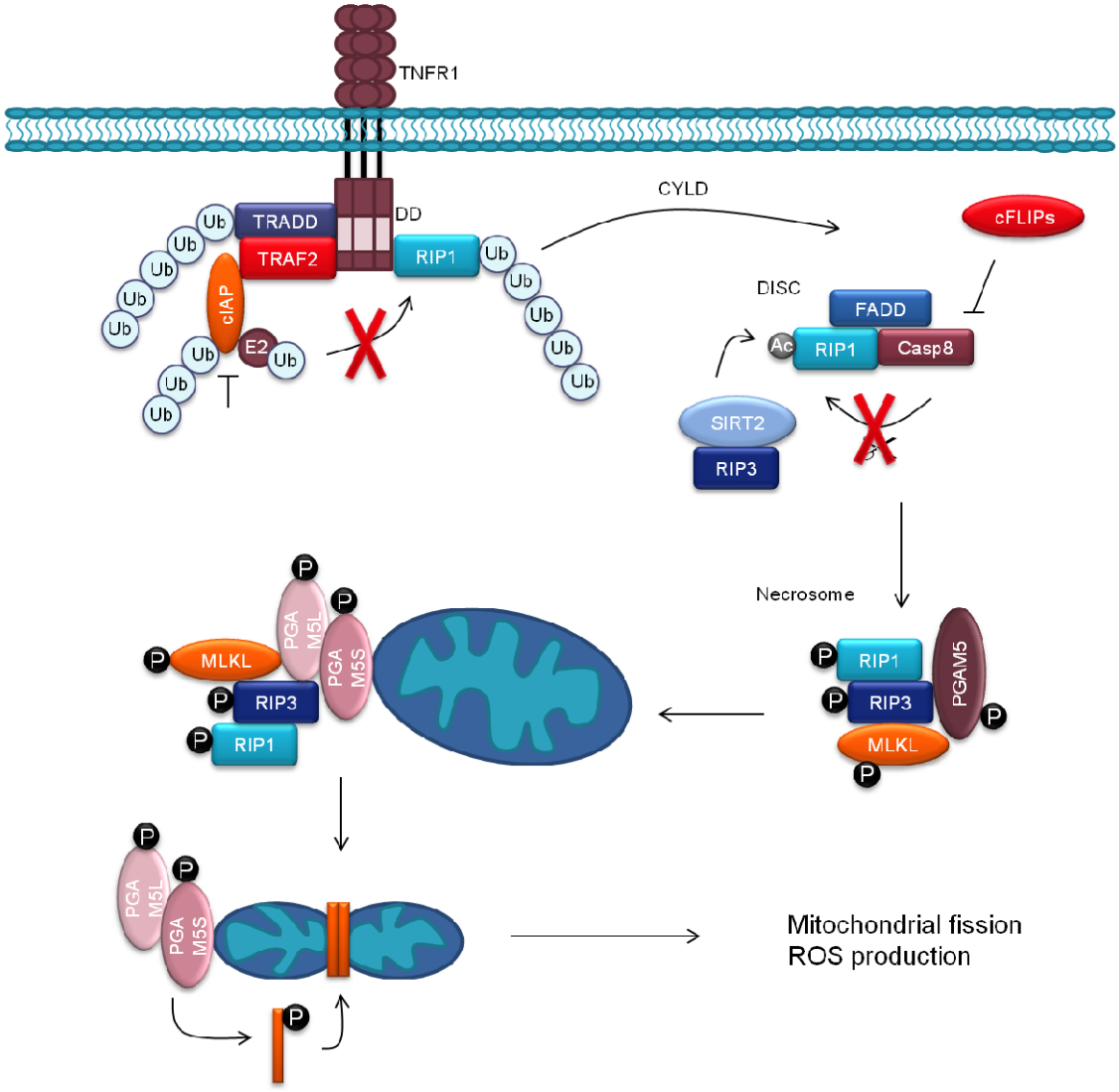


Figure 1.11. Assembly of the necrosome. When caspase8 is downregulated, RIP1 is capable of recruiting RIP3. This process is regulated by the acetylation state of RIP1. Upon deacetylation of RIP1 by SIRT2, RIP1 and RIP3 interact through their RHIM domains and autoactivate. The necrosome then phosphorylates its substrates, including PGAM5 and MLKL. PGAM5S then dephosphorylates Drp-1, which becomes active and promotes mitochondrial fragmentation.

On the other hand, spontaneous formation of a protein module similar to complex II has been described (Feoktistova et al., 2011; Tenev et al., 2011). Due to its independence from DR signaling, this high molecular weight complex comprising RIP1, FADD and caspase-8, among other proteins, was called ripoptosome. The formation of this complex occurs following genotoxic stress (Tenev et al., 2011) or TLR3 stimulation (Feoktistova et al., 2011), both in conditions of cIAP depletion, suggesting that these

proteins inhibit the assembly of the ripoptosome. The $FLIP_L$ isoform is also recruited to the complex, exerting an inhibitory regulation of the ripoptosome. Accordingly, KD of $FLIP_L$ enhanced the formation of the ripoptosome. This complex seems to have the ability to induce both apoptosis and necroptosis, depending on the cellular context (Tenev et al., 2011). Interestingly, $cFLIP_S$ seems to specifically inhibit ripoptosome-induced apoptosis and to promote necroptosis (Feoktistova et al., 2011).

Necroptosis in Disease Context

Necroptosis might be a relevant mechanism in several paradigms of cell death, namely, to fight virus infections. Viruses such as the *Vaccinia* virus express apoptotic inhibitors, delaying the activation of cell death programs in infected T cells, which contributes to disseminate the infection. Thus, if cells are able to activate alternative death programs, the organism may more efficiently fight the infection. Accordingly, *Vaccinia* virus infected T cells are sensitized to TNF-induced necroptotic cell death (Cho et al., 2009). Mouse cytomegaloviruses (MCMV) express cell death suppressors that specifically inhibit RIP3 activity, in a RHIM-dependent manner (Upton et al., 2010). This aspect emphasizes the relevance of necroptosis as a physiological programmed cell death mechanism.

In another disease context, an animal model of cerulean-induced pancreatitis, much of the cell death in the pancreas seems to be attributable to RIP3-dependent necroptosis. This protein is significantly induced specifically in these cells and $RIP3^{-/-}$ animals have a much lesser extent of pancreatic cell death (He et al., 2009).

Necroptosis is also involved in Crohn's disease pathogenesis. Indeed, RIP3 seems to be specifically expressed in the cells that are most affected by the disease, the Paneth cells of the terminal ileum, which might confer increased susceptibility to necroptotic insults. Additionally, intestinal epithelial cells lacking caspase-8 are sensitized to necroptotic cell death, as observed by rescue of this effect with Nec-1. This provides further evidence for a role of caspase-8 in the inhibition of necrosome formation and subsequent activation of necroptosis (Gunther et al., 2011).

Moreover, in a mouse model of cardiac ischemia, Nec-1 provided significant neuroprotection, reducing the infarct size, the phosphorylation of RIP1/3 and the inflammation, while having no impact on the extent of apoptotic cell death (Oerlemans et al., 2012). Likewise, necroptosis was shown to occur in retinal neurons following ischemic damage. Necrostatin-1 was effective in reducing specifically this component

of cell death, without affecting apoptotic neuronal death and in improving the functional outcome in the ischemia challenged retina (Rosenbaum et al., 2010). Also, in a mouse model of retinal detachment, both inhibition of RIP1 as well as RIP3 ablation were shown to be neuroprotective, demonstrating the activation of necroptotic mechanisms upon apoptosis inhibition (Trichonas et al., 2010).

In vitro cerebral ischemia has also been shown to induce a component of necroptotic neuronal death (Xu et al., 2010; Meloni et al., 2011) which was also observed to occur *in vivo*, following neonatal hypoxia/ischemia (Northington et al., 2011) and MCAO (Degterev et al., 2005; Xu et al., 2010). These reports showing a role for necroptosis following ischemic insults, either *in vitro* or *in vivo*, used a specific inhibitor of necroptosis, Nec-1, to support this finding. This inhibitor acts on the kinase activity of RIP1 (Degterev et al., 2008), however, this protein has also been correlated to the apoptotic signaling, mainly as a scaffold for complex II formation. Thus, the specificity of Nec-1 may be argued against, which emphasizes the need to develop novel strategies to clearly dissect the contribution of necroptosis, in order to better clarify the role of this mechanism on neuronal death, following ischemic insults.

Additional neuronal death paradigms have been shown to induce necroptosis. Indeed, following treatment with 24(S)-hydroxycholesterol (24S-OHC), which is cytotoxic, both human neuroblastoma SH-SY5Y cells and cortical neurons were shown to die by necroptosis (Yamanaka et al., 2011). Additionally, menadione, a compound that induces superoxide production, with concomitant mitochondrial dysfunction and ATP depletion, was shown to induce caspase-independent necroptotic like death in HT-22 cells, a cell line of immortalized mouse hippocampal neurons (Fukui et al., 2012). In a mouse model of traumatic brain injury (TBI), Nec-1 was shown to be effective in reducing the damage associated with the injury, suggesting the occurrence of necroptosis (You et al., 2008). Interestingly, necroptosis was also proposed to have a neuroprotective effect by being activated in inflamed microglia, which results in less damage to neurons. Upon inflammatory stimulation, inhibition of caspase-8 activity is beneficial to neurons by promoting necroptosis in microglia, whereas Nec-1 rescues neurodegeneration. This implies that caspase inhibitors could promote neuronal survival against certain insults by promoting microglial demise (Fricker et al., 2013).

Beyond the study of the NMDARs and of the necroptotic neuronal death, we also addressed the activation of effector signaling pathways, such as the MAPKs, which are known to be involved in several neuronal death paradigms.

MAP Kinase Pathways

The MAPKs are a family of stress-responsive proteins that regulate an array of functions in the cell. They exert their effects by phosphorylating a plethora of substrates, including transcription factors, enzymes and cytoskeletal proteins impacting on cell division, proliferation, metabolism and cell fate (Davis, 2000; Qi and Elion, 2005; Burke, 2007).

These proteins have a typical three-tiered activation cascade. Following an appropriate stimulus a MAPK kinase kinase (MAPKKK) autophosphorylates, leading to relief of autoinhibition and oligomerization. The MAPKKK then activates a MAPK kinase (MAPKK), by dual phosphorylation of Ser and Thr residues within the activation loop of the catalytic domain (Songyang et al., 1996; Chen et al., 2001). Finally, the activated MAPKK phosphorylates the MAPK, on conserved Thr and Tyr residues within a conserved Thr-X-Tyr motif on the activation loop (Silva et al., 2005; Burke, 2007). The MAPKKs are highly specific and typically phosphorylate only one MAPK, but are regulated by many, less specific, MAPKKKs. The MAPKs phosphorylate a wide range of substrates on Ser and Thr residues on a consensus PXT/SP motif. The pathway specificity may be increased by the organization of different signaling complexes by distinct scaffold proteins, which have been shown to have many functions, beyond bringing the elements of the pathway together. Scaffold proteins have the ability of binding, sequestering, and fostering the specific components of the pathway. This organization in a multiprotein complex allows for specific types of stimuli to produce unique and coordinated MAPK signaling responses (Burack and Shaw, 2000; Yoshioka, 2004; Xu et al., 2007). The inactivation of the MAPKs is performed by dual-specificity MAPK phosphatases (MKPs) (Keyse, 2000), among other phosphatases. MAPKs may be found in many subcellular locations, namely in the ER, endosomes, microtubules and may translocate to the nucleus to phosphorylate nuclear substrates.

In mammals, three major groups of MAPKs have been defined: ERK, p38 and JNK (Figure 1.12) (Davis, 2000; Johnson and Lapadat, 2002).

EXTRACELLULAR SIGNAL-REGULATED KINASE

The ERK subfamily of MAPKs comprises two members, ERK1 and ERK2, with 44 and 42 kDa that are activated by mitogenic stimuli like growth factors and cytokines.

These kinases regulate cell motility, proliferation, differentiation, meiosis and cell survival and, in neurons, they impact as well on learning and memory processes.

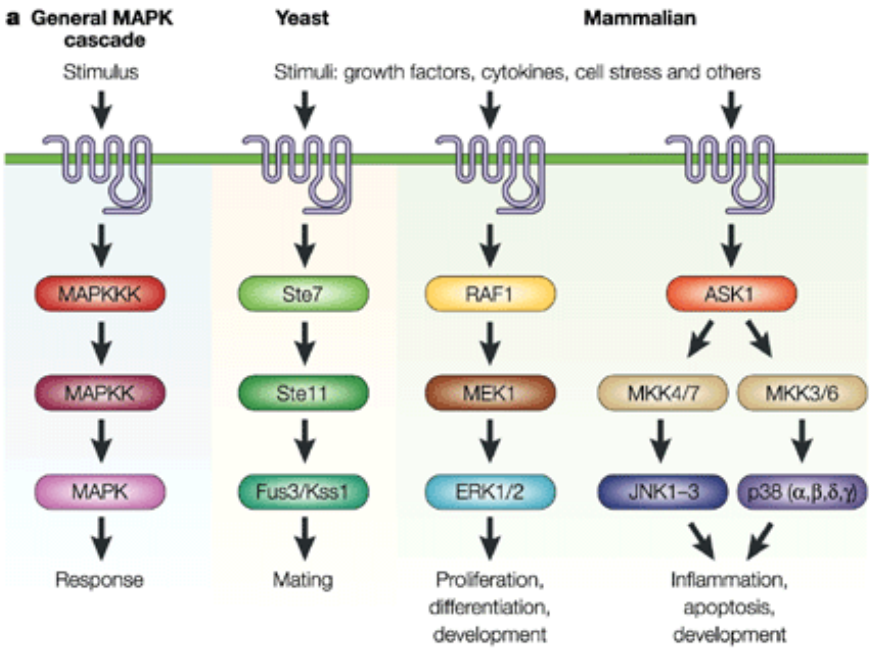


Figure 1.12. MAPK cascades. Diverse stimuli contribute to activation of the typical three-tiered MAPK cascades, consisting of a MAPKKK that activates a MAPKK which then is able to phosphorylate a MAPK. These proteins are involved in an array of cellular functions, in particular the response to stress. (Adapted from Pierce and Lefkowitz, 2001)

The ERK1/2 kinases are phosphorylated in their activation loops by MAPK/ERK kinase 1 (MEK1) and MEK2. Upstream of these MAPKKs are a variety of MAPKKKs, depending on the cellular context and on the stimulus. Raf kinases are the primary MAPKKKs for ERK1/2, but this pathway may also be activated by Mos and Tumor progression locus 2 (TPL2) (Raman et al., 2007). Upon binding of ligands to receptor Tyr kinases or G-protein coupled receptors, Ras becomes activated and triggers the activation of Raf isoforms and their recruitment to the plasma membrane (Wellbrock et al., 2004).

When active, ERK1/2 may phosphorylate a variety of substrates, which include the protein kinases p90 ribosomal S6 kinase (RSK), mitogen and stress activated kinase (MSK) and MAPK interacting kinase (MNK) that have roles in cell attachment and migration. The ERK1/2 proteins also phosphorylate transcription factors, including Elk1, c-Fos, c-Myc and Ets domain factors, among others and the anti-apoptotic protein Bcl-2, which regulates cell survival mechanisms (Johnson and Lapadat, 2002; Yoon

and Seger, 2006). Additionally, ERK phosphorylates the proapoptotic protein Bim, leading to its proteasomal degradation (Luciano et al., 2003; Ley et al., 2004).

The ERK1/2 kinases are distributed throughout the cell, and are associated with plasma membrane receptors and transporters. Association with scaffold proteins is thought to direct ERK1/2 to specific cell compartments, such as the Golgi apparatus and endosomes (Raman et al., 2007).

ERK Activation in the Context of Global Ischemia

Global ischemic insults lead to a reduction in phospho-ERK levels that increase again in the hippocampus, neocortex and striatum, after a short period of reperfusion. The same pattern of phosphorylation is observed for JNK however, after reperfusion, phospho-JNK levels only increase in the hippocampus and striatum (Shackelford and Yeh, 2006). Phospho-ERK1/2 levels appear to be upregulated in the resistant areas of the hippocampus, but not the CA1, which is particularly vulnerable to ischemic insults (Sugino et al., 2000; Zablocka et al., 2003). *In vitro* ischemia also seems to induce ERK phosphorylation in the hippocampus (Bernardi et al., 2010). Additionally, ERK activation seems to be relevant for induction of LTP by *in vitro* ischemia in corticostriatal synapses, in a PKC and mGluR1 dependent manner (Calabresi et al., 2001). The activation of ERK mediated by PKC is thought to mediate neuroprotection in preconditioning models (Jia et al., 2007).

Although ERK signaling is most commonly associated with neuronal survival, some reports describe a role of this signaling pathway in the induction of cell death following OGD. In fact, the ERK1/2-RSK pathway was shown to be activated following OGD and to promote Na^+/H^+ exchanger 1 (NHE1) phosphorylation, which increased its activity and contributed to neuronal death. The toxic role of this cascade was further demonstrated by the neuroprotection afforded by the MEK inhibitor U0126, which is an ERK upstream kinase (Luo et al., 2007).

P38 MAP KINASE

Along with JNK, p38 MAPKs are described as stress-activated protein kinases (SAPKs) because they are frequently activated by a wide range of physical and chemical stresses, namely UV radiation, hormones and cytokines (Figure 1.13). These kinases are involved in diverse cellular processes, such as regulation of the cell cycle,

induction of cell death, differentiation and senescence (Coulthard et al., 2009). This group comprises four isoforms: p38α, β, γ and δ, with significant differences in tissue expression and signaling cascade elements. While p38α and p38β have a ubiquitous expression, the γ and δ isoforms have a more restricted distribution (Raman et al., 2007). The specific function of each p38 isoform is still not completely understood.

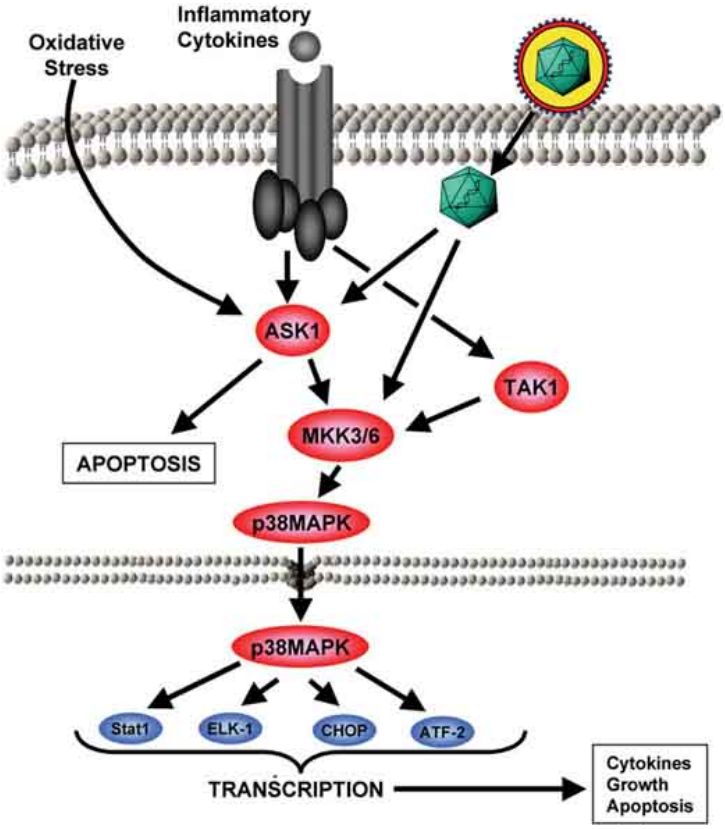


Figure 1.13. p38 MAPK signaling pathway. p38 MAPKs are activated by environmental stress, inflammatory cytokines and virus infection and are dually phosphorylated at the Thr-Gly-Tyr motif by MKK3/6.These are in turn activated by MEKKs, MLKs and ASK1. P38 phosphorylates transcription factors, including Stat1, Elk-1 ATF-2 and CHOP. (Aurelian, 2005)

The p38 MAPKs are regulated by a vast group of MAPKKKs, including MEK kinase (MEKK)1-4, apoptosis signal regulating kinase 1 (ASK1), mixed-lineage kinase (MLK)2/3, TAK1 and TAO kinase (Kataoka et al.)1/2. These MAPKKKs phosphorylate MKK3 and MKK6 and these two MAPKKs are then responsible for dual-phosphorylation of p38 protein on Thr-Gly-Tyr motifs located within the regulatory loop between subdomains VII and VIII (Raman et al., 2007). The kinetics of p38 phosphorylation may be determinant for its functions and sustained p38 activation has

been correlated with induction of apoptosis (Tobiume et al., 2001; Murphy and Blenis, 2006). A role of MKK4 (a JNK pathway MAPKK) in activating p38 was demonstrated *in vitro* and in conditions in which MKK3 and MKK6 are absent (Brancho et al., 2003). Similar to what happens with the other MAPKs, the phosphorylation of p38 is canceled out by phosphatases, including MKPs, protein phosphatase (PP)1 or PP2A in a negative feedback loop that allows tight regulation of p38 activity (Salojin et al., 2006; Hu et al., 2007; Owens and Keyse, 2007).

In line with its diverse functions in cellular function and response to a variety of stress insults, p38 has a wide range of substrates for phosphorylation. These include transcription factors, other kinases and cytoskeletal proteins. Interestingly, the substrate specificities of p38α/β differ from that of p38γ/δ. For example, MAPK-activated protein kinase 2 (MAPKAPK2) is specifically phosphorylated by the α/β isoforms, but not by p38γ/δ (Raman et al., 2007) and stathmin, a cytoplasmic protein involved in microtubule dynamics, is specifically phosphorylated by the p38δ isoform (Parker et al., 1998).

This pathway may also be regulated by scaffold proteins, such as JIP2/4 (Buchsbaum et al., 2002; Kelkar et al., 2005), TAB1 (in a MAPKK-independent manner) (Ge et al., 2002) and the osmosensing scaffold for MEKK3 (OSM) (Uhlik et al., 2003), although the impact of scaffold proteins on p38 signaling is much less studied than their role on the JNK pathway.

The Role of p38 in Disease

The p38 MAPK has prominent roles in diverse cell death paradigms and is important in the etiology of several human disorders (Coulthard et al., 2009), including those with an inflammatory content (Kumar et al., 2003). These include rheumatoid arthritis (Korb et al., 2006; Diarra et al., 2007), Crohn's disease (Bantel et al., 2002; Waetzig et al., 2003) and psoriasis (Johansen et al., 2005; Yu et al., 2007), all of which display increased p38 activity in the affected cells. The p38 MAPK has also been implicated in a plethora of neurological disorders, including AD, HD, Parkinson's disease (PD), amyotrophic lateral sclerosis (Ivanov et al.) and MS (Harper and Wilkie, 2003; Johnson and Bailey, 2003). Additionally, some p38 substrates, like MAPKAPK2 (Culbert et al., 2006; Thomas et al., 2008b) and activator transcription factor 2 (ATF2) (Johnson and Bailey, 2003), have been associated with the development of

neurodegenerative disorders. Inhibition of p38 MAPK is thought of as a promising avenue towards treatment to some of these disorders (Bendotti et al., 2006).

p38 MAPK Activation in Cerebral Ischemia

Activation of p38 MAPK has been reported to correlate with induction of cell death following global ischemia since administration of SB203580, a specific p38 inhibitor, had a neuroprotective effect, while inhibiting phosphorylation of known p38 substrates, such as the transcription factor ATF2 (Sugino et al., 2000). This suggests that sustained p38 activation can have a pro-death role, thereby contributing to the selective vulnerability of the CA1 area of the hippocampus.

The OGD challenge also activates the p38 signaling pathway (Guo and Bhat, 2007; Shinozaki et al., 2007; Xu et al., 2009; Lu et al., 2011), which has death promoting effects in organotypic hippocampal slices (Barone et al., 2001; Strassburger et al., 2008; Lu et al., 2011) and in forebrain cultures (Legos et al., 2002), since neuronal death is reversed in the presence of the p38 inhibitors, SB239063 or SB203580. Pharmacological inhibition of this kinase also reduces the extent of neuronal death following *in vivo* focal ischemia (Barone et al., 2001). Knock-down of p38 also has a neuroprotective effect, both in organotypic hippocampal slices, reducing the formation of superoxide induced by OGD (Lu et al., 2011), as well as in a motor neuron cell line (Guo and Bhat, 2007). The p38 MAPK mediates the inflammatory response, as SB239063 reduces the extent of activated microglia following OGD, but also the IL-1β mRNA levels (Strassburger et al., 2008). The p38 MAPK was also found to lead to activation of NADPH oxidase, thereby contributing to oxidative stress and subsequent neuronal death induced by OGD and by an *in vivo* model of neonatal hypoxia-ischemia (Lu et al., 2012). Additionally, inhibition of the interaction between nNOS and NOS1 associated protein (NOS1AP), which leads to p38 activation, confers neuroprotection against neonatal hypoxia-ischemia (Li et al., 2013).

The p38 signaling pathway can be regulated by calpains, which are Ca^{2+}-dependent proteases with a determinant role in ischemic conditions, as previously mentioned. Calpains cleave a plethora of substrates, as is the case of the phosphatase STEP. The FL STEP61 is cleaved to a smaller isoform, STEP33, in a NMDAR-dependent manner. Upon extrasynaptic NMDAR activation, p-p38 levels are increased with concomitant cleavage of STEP61 by calpain, in a process that is facilitated by Cdk5. Thus, STEP seems to counteract the toxic effects of NMDAR overactivation by

dephosphorylating key kinases such as p38, thereby improving neuronal survival. Accordingly, a TAT peptide with the ability to inhibit STEP cleavage had neuroprotective effects against the OGD challenge in corticostriatal slices. This neuroprotective effect is mediated by a decrease in p38 phosphorylation induced by OGD (Xu et al., 2009). Global ischemic insults induce strong expression of p25 in CA1 neurons, which is sufficient to activate Cdk5 in this area and induce subsequent neuronal death. The Cdk5-mediated induction of neuronal death seems to derive from phosphorylation of GluN2A subunits of NMDARs (Ser1232), which increases NMDAR electrophysiological properties. Additionally, AMPARs seem to be the route of Ca^{2+} entry, leading to calpain activation which, in turn, cleaves p35 to produce p25 that then activates Cdk5. This effect is specific to the CA1, as the CA3 and the DG do not display such induction (Wang et al., 2003; Liu et al., 2004).

C-JUN N-TERMINAL KINASE

The JNK MAPKs, also known as SAPKs, are activated by a wide range of environmental stresses (Kyriakis and Avruch, 1990; Kyriakis et al., 1994; Davis, 2000; Weston and Davis, 2002), but are also involved in physiological functions (Waetzig et al., 2006). Accordingly, JNK is activated in response to cytokines (Shirakabe et al., 1997; Yao et al., 1999), DNA damage and oxidative stress (Finkel and Holbrook, 2000; Kamata et al., 2005), UV radiation (Derijard et al., 1994) and excitotoxicity (Yang et al., 1997; Maroney et al., 1999; Vieira et al., 2010), but is also important during embryonic development (Boylan and Gruppuso, 1996; Sun et al., 2013) and for neurite outgrowth (Yao et al., 1997; Tararuk et al., 2006; Feltrin et al., 2012; Tezuka et al., 2013).

Three genes encode mammalian JNK isoforms, *Jnk1*, *Jnk2* and *Jnk3*. These genes are alternatively spliced to produce ten different isoforms of 46 and 54 kDa (Gupta et al., 1996) and while JNK1 and JNK2 are ubiquitously expressed, JNK3 expression is restricted to the brain, heart and testis (Mohit et al., 1995; Martin et al., 1996). Some studies using *Jnk* gene disruption in mice demonstrated that there is an extensive complementation between *Jnk* genes and that individual tissues may express specific isoforms of JNK (Tournier et al., 2000).

The signaling through the JNK group is highly complex, due to a variety of MAPKKKs that regulate JNK activity, including MLKs, ASK and MEKK1-4. These MAPKKKs activate two MAPKKs, MKK4 and MKK7, which are then responsible for phosphorylating JNK isoforms (Figure 1.14) (Davis, 2000; Weston and Davis, 2002;

Weston and Davis, 2007). In general, MKK7 is primarily activated by cytokines (such as TNFα and IL-1) and MKK4 by environmental stress. Although both MAPKKs can act alone to phosphorylate JNK on Tyr and Thr, and therefore activate it, they can act synergistically to activate JNK in response to certain stimuli (Davis, 2000; Burke, 2007). In general, MKK4 preferentially targets JNK Tyr-185, whereas MKK7 usually phosphorylates Thr-183 (Silva et al., 2005).

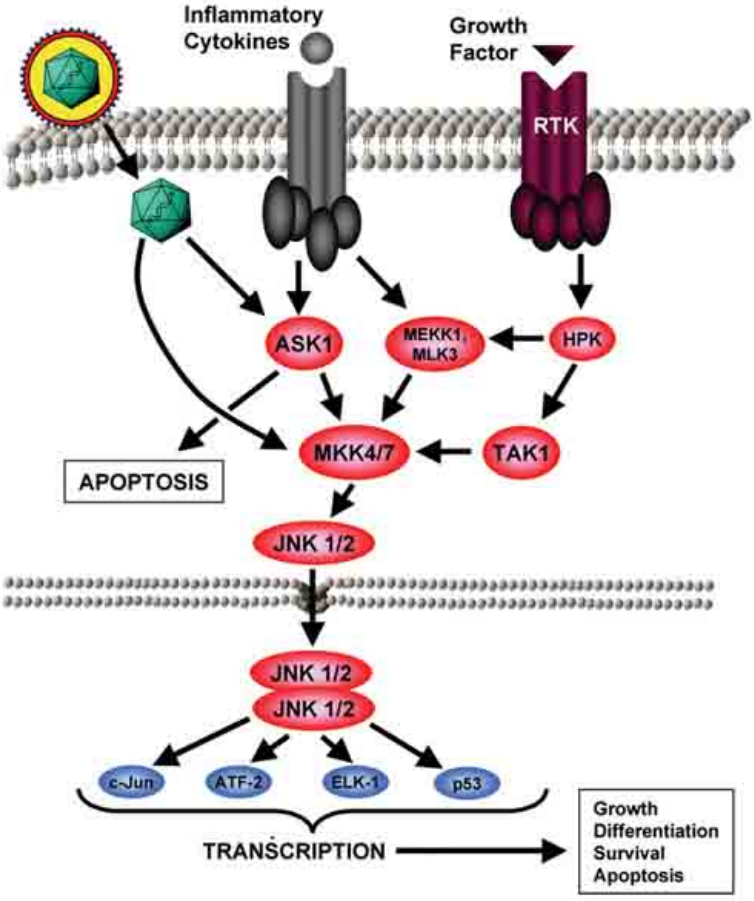


Figure 1.14. JNK signaling pathway. JNK is activated by stress signals, namely heat shock, osmotic stress and pro-inflammatory cytokines. MKK4 and MKK7 phosphorylate JNK (at Thr-Pro-Tyr motif). MEKKs 1-4, MLK3 and ASK-1, in turn, phosphorylate MKK4/7. Activated JNKs dimerize and translocate to the nucleus where they phosphorylate transcription factors including c-Jun, ATF-2, Elk-1 and p53. Activation of the JNK signaling cascade generally results in apoptosis and is involved in inflammation. (Aurelian, 2005)

The existence of a large number of MAPKKKs that activate the JNK pathway suggests that different MAPKKKs respond to distinct stimuli, thereby conferring specificity to JNK signaling (Weston and Davis, 2007). Accordingly, whilst TAK1 was

shown to activate JNK in response to inflammatory cytokines and TLR activity (Sato et al., 2005; Shim et al., 2005; Wan et al., 2006), MEKK3 is determinant for TLR-8-induced JNK activation (Qin et al., 2006) and MLK3 and TPL2 were demonstrated to contribute to TNF-mediated JNK signaling in embryonic fibroblasts (Brancho et al., 2005; Das et al., 2005). The kinase MLK3 is also important for the response to metabolic stress induced by free fatty acids (Jaeschke and Davis, 2007) and, in neurons, for nerve growth factor (NGF) withdrawal-induced cell death (Xu et al., 2001).

The JNK cascade may be regulated by a variety of scaffold proteins (Morrison and Davis, 2003), including members of the JNK-interacting protein (JIP) family (Whitmarsh et al., 1998; Yasuda et al., 1999; Kennedy et al., 2007), β-arrestin2 (McDonald et al., 2000) and plenty of SH3s (POSH) (Xu et al., 2003; Xu et al., 2005; Wilhelm et al., 2007). These scaffolds may transport the signaling module to specific locations in the cell, which may confer a higher specificity to signaling via this pathway.

The kinase JNK has the ability to phosphorylate a plethora of substrates in a stimulus-dependent manner. The archetypal JNK substrate is the transcription factor c-Jun, which is dual-phosphorylated by JNK in its N-terminal domain (Ser63 and Ser73), increasing its transcriptional activity (Pulverer et al., 1991; Derijard et al., 1994; Minden et al., 1994; Watson et al., 1998). Besides c-Jun, JNK also phosphorylates other transcription factors, namely of the Jun, Fos and ATF families (for example, JunB, JunD, c-Fos and ATF2). Members of the Jun and Fos families assemble the AP-1 complex (Bogoyevitch and Kobe, 2006), which is responsible for the transcription of several stress responsive genes. Furthermore, JNK contributes to the mitochondrial pathway of apoptosis by inducing the gene expression of mitochondrial proteins like Bim (Harris and Johnson, 2001; Putcha et al., 2001; Whitfield et al., 2001; Gilley et al., 2003). This kinase has been shown to phosphorylate H2A histone family, member X (H2AX), which is determinant for DNA fragmentation induced by apoptotic stimulation. Phosphorylation mutants for Ser139 resulted in an absence of DNA fragmentation upon UV radiation (Lu et al., 2006; Sluss and Davis, 2006). Despite its major role in gene transcription regulation, JNK can also mediate apoptotic mechanisms via non-transcriptional effects (Tournier et al., 2000; Bogoyevitch and Kobe, 2006). Accordingly, several anti- and pro-apoptotic proteins of the Bcl-2 family are JNK substrates and their phosphorylation by this kinase regulates their activity in the apoptotic signaling (Maundrell et al., 1997; Yamamoto et al., 1999; Kharbanda et al., 2000; Donovan et al., 2002; Lei and Davis, 2003; Liu and Lin, 2005). JNK also has some neuron-specific substrates, namely two AMPAR subunits – GluA4 and GluA2L. Phosphorylation by JNK contributes to the regulation of AMPARs containing these

subunits, which are constitutively phosphorylated by JNK at GluA4-Thr855 and GluR2L-Thr912. This phosphorylation is proposed to play a role in controlling activity-dependent GluR2L trafficking (Thomas et al., 2008a). JNK mediated phosphorylation of GluA4 on Thr855 is increased upon excitotoxic stimulation however, the role of this phosphorylation in this context is not fully understood (Vieira et al., 2010).

The activity of JNK is counteracted by MKPs, as demonstrated by increased JNK activity upon ablation of either MKP1 (Chi et al., 2006; Wu et al., 2006b; Zhao et al., 2006) or MKP5 (Zhang et al., 2004). Interestingly, the association of the MKPs may occur directly with JNK or via interaction with the JNK scaffold proteins JIP1 and β-arrestin2 (Willoughby et al., 2003; Willoughby and Collins, 2005). The phosphatase MKP1 is part of a negative feedback loop in a NGF withdrawal paradigm of cell death in sympathetic neurons. Activation of JNK signaling promotes MKP1 expression via c-Jun/ATF2 transcriptional activity (Kristiansen et al., 2010).

The role of JNK in apoptosis is well established (Eilers et al., 1998; Tournier et al., 2000; Tournier et al., 2001), however it seems to be highly dependent on the type of cell and on the specific stimulus (Davis, 2000; Weston and Davis, 2002). Notably, the requirement for JNK and p38, in neuronal death paradigms, seems to be highly stimulus-specific. For example, whilst caspase-independent glutamate-induced neuronal demise relies on p38 activity, trophic factor withdrawal is solely dependent on JNK signaling (Cao et al., 2004b). Additionally, JNK seems to have dual roles in the decision between life and death. In fact, JNK has been reported to have two phases of activity (Hirt et al., 2004; Ventura et al., 2006; Vieira et al., 2010), which may have differential impact on the cellular fate. An initial, transient increase in JNK activity was shown to contribute to cell survival whereas a later, sustained phase of JNK activity reportedly contributed to cell death (Ventura et al., 2006). Interestingly, neurons were shown to have distinct pools of JNK, performing individual functions. A pool located close to the neurites was proposed to be responsible for physiological functions while a nuclear pool of JNK, more readily available to phosphorylate c-Jun, was responsible for the cell death mechanisms (Coffey et al., 2000; Bjorkblom et al., 2008). Furthermore, neurons have a high constitutive JNK activity, attributed in its majority to JNK1 and, upon stressful insults, JNK2/3 is activated and is responsible for the damaging effects of c-Jun (Coffey et al., 2002). Furthermore, although JNK clearly contributes to apoptotic cell death, it has also been shown to be involved in other cell death mechanisms, namely necrosis (Ventura et al., 2004).

JNK Signaling and Disease

As a hallmark signaling pathway in stress responses, JNK activity has been associated with a variety of pathologies, especially those with a strong apoptotic component (Cui et al., 2007b). Indeed, the JNK signaling pathway is involved in the etiology of Type 2 diabetes (Hirosumi et al., 2002; Jaeschke et al., 2005; Kaneto et al., 2006), cancer and inflammatory diseases. Furthermore, JNK activity is critically involved in several neurological disorders, including PD (Saporito et al., 2000; Hunot et al., 2004; Silva et al., 2005), polyglutamine diseases (Liu, 1998; Taylor et al., 2013), AD (Savage et al., 2002; Ferrer et al., 2005) and cerebral ischemia (Herdegen et al., 1998; Kuan et al., 2003; White et al., 2012).

In order to counteract the toxic effects of JNK, several compounds have been developed, with different specificities. One of the most promising is a peptide inhibitor, which consists of a small peptide corresponding to the JNK binding domain (JBD) of the JNK scaffold JIP-1, attached to the cell permeable TAT sequence (D-JNKI-1). This peptide acts as a competitive inhibitor, preventing the binding of JNK to its substrates that possess a similar JBD. This peptide has been shown to have protective effects against IL-1β-induced apoptosis in pancreatic β-cells (Bonny et al., 2001) and in several models of focal ischemia (Borsello et al., 2003). Notably, inhibition of JNK, and also p38, is a promising avenue for treatment against a wide range of neurological disorders (Bendotti et al., 2006).

JNK and Global Ischemia

Global ischemic insults lead to the activation of MAPK signaling pathways: JNK, p38 and ERK (Ozawa et al., 1999; Sugino et al., 2000). The pattern of MAPK activation seems to be dependent on the intensity and duration of the stimulus and also on the animal model used. Hence, the timing of MAPK activation varies in different reports. Indeed, while Sugino and coworkers observed activation of JNK and p38 both in the CA1 and CA3 areas of the hippocampus, although with differences in terms of kinetics (Sugino et al., 2000), other authors have reported a specific activation of JNK in the CA1 area. These authors describe a biphasic activation of the JNK pathway in CA1, accompanied by c-Jun phosphorylation (Zablocka et al., 2003), involving the upstream kinases ASK1 and MKK4, whereas the CA3 and DG do not display JNK activation. Interestingly, only the second phase of JNK pathway activation seems to be specifically mediated by the signaling complex formed by ASK1-MKK4-JNK, which may influence

the role of JNK on the neuronal outcome. Also, this second phase seems to be mediated by Ca^{2+}-permeable AMPARs (CP-AMPARs) and oxidative stress (Zhang et al., 2003). The AS601245 compound, a specific JNK inhibitor, was shown to be neuroprotective against neuronal death in the hippocampus after global ischemia in gerbils and focal ischemia in rats. Moreover, this inhibitor reverses the increased c-Jun phosphorylation observed in the CA1 area after the insult (Carboni et al., 2004). In view with the involvement of CP-AMPARs in the activation of JNK, we have shown that overactivation of homomeric GluA4-containing AMPARs induced JNK activation, in a biphasic and Ca^{2+}-dependent manner (Vieira et al., 2010). Moreover endogenous GluA4 of the rat brain hippocampus associates with JIP1 upon excitotoxic stimulation *in vivo*, suggesting that the JIP1 scaffold couples CP-AMPARs to JNK activation (Vieira et al., 2010) and to increased AP-1 binding activity (Santos et al., 2006) propagating the excitotoxic signal from the cell surface to the nucleus. The OGD-induced death has also been shown to be mediated by JNK signaling. In fact, there are reports of increased c-Jun phosphorylation, following OGD stimulation (Thevenet et al., 2009) as well as of JNK itself, which corresponds to increased activation of this kinase (Shinozaki et al., 2007). This increase in c-Jun phosphorylation seems to be related with induction of CA1 hippocampal neuron death. Additionally, some reports show significant neuroprotection afforded by the JNK inhibitor D-JNKI-1, against the OGD challenge (Hirt et al., 2004; Benakis et al., 2010). In fact, D-JNKI-1 has elicited promising results, since administration 6h after OGD had a neuroprotective effect and improved neuronal functional recovery. Interestingly, administration at 3h after OGD had no significant effect, which points to dual functions performed by JNK: a pro-survival, and a pro-death role. *In vivo*, D-JNKI-1 also proved neuroprotective. When administered 3h after permanent MCAO, it reduced the infarct volumes and improved neurological scores (Hirt et al., 2004) and reduced c-Jun phosphorylation (Repici et al., 2007). This peptide was also effective in preventing NMDA-induced excitotoxicity (Centeno et al., 2007). Another JNK inhibitor peptide, TAT-TIJIP, was also found to be protective against OGD and to prevent caspase-3 activation in this paradigm of neuronal death (Arthur et al., 2007). In $JNK3^{-/-}$ hippocampal neurons, there is increased resistance to OGD-induced death and they display reduced cytoplasmic staining for cytochrome c. $JNK3^{-/-}$ animals also display reduced vulnerability to excitotoxicity (Yang et al., 1997) and hypoxia-ishemia accompanied by decreased levels of apoptotic markers, such as Bim and Fas (Kuan et al., 2003).

Some JNK upstream kinases, such as TAK1, have been shown to be activated in neurons, in response to OGD. This MAPKKK is related to neuronal death as its

acute inhibition protects cortical neurons from OGD-induced apoptosis, reduces the infarct volume and improves the sensorimotor outcome following MCAO. Activation of TAK1 results in phosphorylation and activation of the MAPKs JNK and p38 following OGD, which then induce neuronal death, through the transcriptional activity of AP-1 and NF-κB, which contributes to oxidative stress generation. Interestingly, prolonged TAK1 inhibition/deletion is not neuroprotective as other MAPKKKs, such as ASK1, compensate for TAK1 absence (Neubert et al., 2011).

JNK also seems to be correlated to the toxic effects of KAR activation during *in vitro* or *in vivo* ischemia. Indeed, the signaling module GluK2-PSD95-MLK3 seems to induce toxicity in rat hippocampal neurons following OGD and 4-VO (Hu et al., 2009) or MCAO (Yu et al., 2009). This signaling module leads to downstream JNK3 activation, with subsequent c-Jun phosphorylation and activation of the apoptotic machinery. By interfering with the formation of this module, increased neuroprotection is observed following *in vitro* and *in vivo* ischemia (Pei et al., 2006; Hu et al., 2009; Yu et al., 2009).

Although many reports demonstrate the importance of MAPKs to the death promoting effects following OGD or *in vivo* ischemia, some reports challenge this view by showing no significant protection afforded by MAPK inhibitors. Zhou et al. describe a reduction in ERK1/2, p38 and JNK phosphorylation following OGD, and no significant effect of the MEK inhibitor U0126 or SB203580, a p38 inhibitor, both in young (P10) and adult (2 month old) rat hippocampal slices (Zhou et al., 2007). These discrepancies in the reports may be justified by the timing of application of the inhibitors, which varies in the different reports, as well as the intensity of the stimuli.

The transcription factor AP-1 is composed of dimers of proteins of the Jun and Fos families, among others, that are regulated by phosphorylation mediated by MAPK protein members. The c-Jun transcription factor has a role in the induction of neuronal death following OGD. In fact, KD of this protein significantly protects neurons in this model of neuronal death (Cardoso et al., 2010). Excitotoxic stimulation of CP-AMPARs leads to increased DNA binding activity of AP-1, and the inhibition of c-Jun transcriptional activity was cytoprotective (Santos et al., 2006). Similar to the JNK inhibitory peptides, AP-1 inhibition using TAT peptides significantly protects cortical neurons against an excitotoxic paradigm (Meade et al., 2010).

Overall, both JNK and p38 MAPKs have an established role in the induction of neuronal death following cerebral ischemic insults and targeting these pathways is a promising avenue for the development of novel strategic therapies for this type of pathology.

Objectives

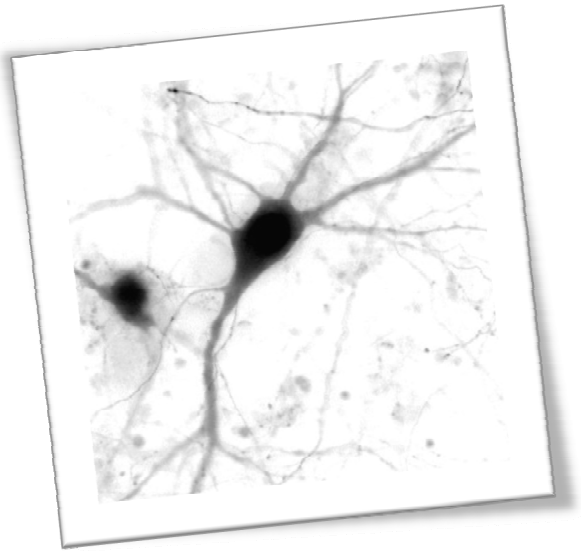
In this work, we aimed to study the mechanisms of toxicity underlying the neuronal death induced by OGD in rat primary cultures of hippocampal neurons, an *in vitro* global ischemia model, in order to advance the knowledge of the molecular mechanisms of cerebral ischemia, thus contributing to identify new molecular targets that may be useful for the development of effective therapeutic strategies to this pathology.

To achieve our main goal, we targeted different steps in the mechanisms that lead to neuronal death. Hence, we investigated the activation of NMDARs and the specific contribution of the GluN2B subunit to the toxicity induced by the OGD challenge. For this purpose, we used a GluN2B$^{-/-}$ culture system and addressed the contribution of the C-terminal domain of this subunit by introducing different mutant GluN2B subunits in these neurons. With this, we intended to clarify the domains in the C-terminus of GluN2B responsible for the toxicity of this subunit upon OGD (Chapter II). We also investigated the activation of a novel type of programmed cell death called necroptosis upon OGD stimulation, in order to establish the underlying mechanisms that lead to the activation of this cell death program under ischemic stimulation. For this we analysed the neuroprotective effect of Nec-1 on the OGD-induced death, using a few cell death assays (LDH, PI uptake and analysis of the nuclear morphology with Hoechst 33342). We also studied the contribution of the necrosome proteins RIP1 and RIP3 to the neuronal death process following OGD (Chapter III). Additionally, we addressed the study of signaling pathways commonly associated with ischemic neuronal death, specifically the MAPK signaling pathways JNK and p38. We analysed the activation of both pathways and observed the effect of the inhibition of either p38 or JNK to the hippocampal neuronal death elicited by OGD: Additionally, we assessed the mRNA levels of some MAPK substrates and upstream kinases using qPCR (Chapter IV).

Our approach aimed to clarify the events associated with different players of the neuronal death process under ischemic stimulation so as to pinpoint molecular targets involved in hippocampal neuronal death. Ultimately, this will contribute to a better understanding of the molecular mechanisms that contribute to neurodegeneration in cerebral ischemia and, hopefully, will be valuable in the development of therapeutics to improve patient outcome.

Chapter II

Diverse Domains in the C-terminus of the GluN2B Subunit of NMDARs Contribute to Neuronal Death upon *in vitro* Ischemia



Abstract

Global cerebral ischemia induces selective neurodegeneration of specific subsets of neurons throughout the brain, namely in the Cornu Ammonis 1 (CA1) region of the hippocampus and in the cortical layers II, V and VI. One of the major hallmarks of cerebral ischemia is a phenomenon called excitotoxicity. It consists in the overactivation of glutamate receptors (GluRs) at the postsynaptic membrane with consequent overload of Ca^{2+}. This leads to deregulation of Ca^{2+} homeostasis and neuronal demise. N-methyl-D-aspartate receptors (NMDARs) are considered to be largely responsible for excitotoxic mechanisms due to their high Ca^{2+} permeability. The NMDARs are most prominently composed of combinations of two GluN1 subunits and two GluN2A or GluN2B subunits. While GluN2A is commonly believed to be located at the synapse and to mediate prosurvival signaling, GluN2B is thought to be most abundant at extrasynaptic sites and its activation is believed to mediate neurotoxicity. We investigated whether GluN2B is in fact responsible for activation of prodeath signaling using an *in vitro* model of global ischemia by submitting primary cultures of hippocampal neurons to oxygen and glucose deprivation (OGD). We observed that OGD induces a component of excitotoxic cell death that is prevented upon GluN2B antagonism or in the absence of this subunit. We also describe a crucial role of the C-terminal domain of GluN2B that goes beyond the PSD95/Dlg/ZO-1 (PDZ) domain, which has been previously established. Indeed, expression of YFP-GluN2B in GluN2B$^{-/-}$ mouse cortical neurons rescued neuronal death upon OGD, whereas expression of the YFP-GluN2B C-terminus mutants for the binding sites to postsynaptic density 95 (PSD95), Ca^{2+}-calmodulin kinase II (CaMKII) or clathrin adaptor protein 2 (AP2) failed to induce neuronal death in OGD conditions. Taken together, our results show a crucial role of the C-terminal domain of the GluN2B subunits in promoting neuronal death in ischemic conditions, which seems to be responsible for the divergent roles of the GluN2A and GluN2B subunits to neuronal fate.

Introduction

Transient cerebral global ischemia is one of the most common causes of disability and mortality in the western world. It results from a lack of blood supply to the entire brain that may arise due to cardiac arrest, leading to the delayed death of specific subpopulations of neurons. The hippocampus and some cerebral cortical

layers are prominently affected following global cerebral ischemia in patients (Petito et al., 1987) and also in animal models (Kirino, 1982; Zukin et al., 2004). Global cerebral ischemic insults can be reproduced *in vitro* by performing oxygen and glucose deprivation (OGD) on primary neuronal cultures or brain slices, typically from the hippocampus or the cerebral cortex (Goldberg and Choi, 1993; Martin et al., 1994; Calderone et al., 2003).

Excitotoxicity is a major hallmark of several neurological disorders, including cerebral ischemia, and is characterized by the accumulation of glutamate in the extracellular space, leading to overactivation of glutamate receptors (GluRs) and the consequent induction of neuronal death. One key factor in excitotoxic cell death is the Ca^{2+} overload (Arundine and Tymianski, 2003; Forder and Tymianski, 2009). Among GluRs, the N-methyl-D-aspartate receptors (NMDARs) have long been implicated in excitotoxic phenomena, due to their high Ca^{2+} permeability (Goldberg and Choi, 1993; Gwag et al., 1995; Wang and Qin, 2010). The NMDARs are ionotropic GluRs (iGluRs) that form tetrameric structures assembled from two obligatory GluN1 subunits and two GluN2 (A-D) or GluN3 (A, B) subunits (Traynelis et al., 2010). Most NMDAR complexes are composed by GluN2A or GluN2B in combination with GluN1 subunits. In rodents, the GluN2 subunits are differentially regulated throughout development, with GluN2B being highly expressed during embryonic development and GluN2A expression increasing around the second post-natal week (Watanabe et al., 1992; Monyer et al., 1994). The role of these two subunits in excitotoxicity has remained controversial, as some reports suggest that both subunits contribute to neuronal death (Stanika et al., 2009; Zhou et al., 2013), while others imply a differential contribution of the two subunits (Wyllie et al., 2013). Specifically, GluN2A is thought to be related with activation of survival signaling (Liu et al., 2007; Terasaki et al., 2010), while GluN2B activates deleterious pathways (Aarts et al., 2002; Soriano et al., 2008; Martel et al., 2012).

In this work we investigated the impact of the GluN2B subunit to OGD-induced neuronal death. We chose this insult because most reports use excitotoxic treatments (usually high NMDA concentrations), which do not correlate as accurately to *in vivo* cerebral ischemia as the OGD challenge. We observed a specific role of this subunit in OGD-induced death, in particular of its C-terminal domain. While GluN2B expression in $GluN2B^{-/-}$ cortical neurons rescued neuronal death in ischemic conditions, the GluN2B subunit with its C-terminal tail swapped with that of GluN2A failed to produce cell death upon OGD. We confirmed previous evidence showing a neuroprotective effect of interfering with the interaction between GluN2B and postsynaptic density 95 (PSD95)

but we went further to observe a determinant role of two additional domains in the C-terminus of the GluN2B subunit. Indeed, we report the absence of neuronal death in the presence of GluN2B mutant subunits for the Ca^{2+}-calmodulin kinase II (CaMKII)- and clathrin adaptor protein (AP2)-binding sites.

Materials & Methods

Materials

The NMDAR antagonist (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801, #0924) and the GluN2B selective antagonist ifenprodil (#2892) were purchased from Tocris Bioscience (Bristol, UK).

The plasmids pll-synapsin-GluN2B-YFP, encoding the wild-type (WT) GluN2B subunit of NMDARs, pll-synapsin-GluN2B swap tail-YFP, encoding the GluN2B subunit with the entire tail swapped with that of GluN2A, pll-synapsin-GluN2B PDZ mutant-YFP, encoding the GluN2B subunit with a mutation on the PDZ domain (ESDV → EAAV), pll-synapsin-GluN2B CaMKII mutant-YFP, encoding the GluN2B subunit with a mutation on the CaMKII binding domain (RQHS → QQHD) and pll-synapsin-GluN2B AP2 mutant-YFP, encoding the GluN2B subunit with a mutation on the AP2 binding domain (YEKL → ASKL) (Figure 2.2-B) were generous gifts from Ann Marie Craig, University of British Columbia, Vancouver, Canada.

The TAT cell permeable peptides L-TAT and L-TAT-$NR2B_{9c}$ were generous gifts from Michael Courtney, Kuopio University, Kuopio, Finland.

Cell Culture and Transfection

Two distinct types of neuronal cultures were used in this study. We used cultured cortical neurons from $GluN2B^{-/-}$ and WT littermates (due to limiting numbers of $GluN2B^{-/-}$ hippocampal neurons), or primary cultures of rat hippocampal neurons (for the studies with NMDAR antagonists).

For the $GluN2B^{-/-}$ and WT littermate cultures, cortical neurons were prepared from embryonic day 17-18 (E17-18) mice, as previously described (Ferreira et al.,

2011). Briefly, cortices were dissected and maintained in Hibernate E (Brain Bits, Springfield, IL) supplemented with NeuroCult™ SM1 Neuronal Supplement (StemCell™ Technologies, Grenoble, France) at 4°C overnight, while genotyping was performed. Tissues from the same genotype were pooled together and dissociated with papain (20 units/ml, 10 min, 37°C) and deoxyribonuclease I (0.20 mg/ml).

Cortical neurons were electroporated on the day of the culture using the AMAXA system (Lonza, Basel, Switzerland). Briefly, 0.75 million cells were centrifuged and the cell pellet resuspended in 50 µl of Ingenio™ Electroporation Solution (MIR50111 – Mirus Bio LLC, Madison, WI) and 2.5 µg of each plasmid was added. After electroporation, neurons were transferred to coverslips coated with poly-D-lysine (PDL) in 12 multiwell (MW) plates containing pre-warmed minimal essential medium (MEM) supplemented with 10% horse serum. Once the neurons attached to the substrate, the plating medium was removed and neurons were placed in Neurobasal® Medium (Gibco®, Life Technologies™, Paisley, UK) supplemented with 2% NeuroCult® SM1, 0.5 mM glutamine, 0.125 µg/ml gentamicin, and insulin (20 µg/ml). Neurons were maintained at 37°C in a humidified incubator with 5% CO_2/95% air for 15 days *in vitro* (DIV).

For neuroprotection studies with NMDAR antagonists, primary cultures of hippocampal neurons were prepared from E18 rats (Santos and Duarte, 2008). Briefly, hippocampi were dissected and dissociated with trypsin (1.5 mg/ml Hank's Balanced Salt Solution – HBSS). Neurons were cultured in MW plates with or without glass coverslips, coated with PDL, at a density of 85,000 cells/cm^2, in Neurobasal® medium supplemented with 2% NeuroCult™ SM1 Neuronal Supplement, 0.5 mM glutamine, 0.125 µg/ml gentamicin and 25 µM glutamate. Neuronal cultures were maintained at 37ºC in a humidified incubator with 5% CO_2/95% air up to 15 DIV.

Oxygen-Glucose Deprivation (OGD) Challenge

At DIV 15, hippocampal neurons were challenged with an *in vitro* ischemic insult. For that purpose, neurons were placed in a glucose-free deoxygenated buffer medium (OGD medium – 10 mM HEPES, 116 mM NaCl, 5.4 mM KCl, 0.8 mM NaH_2PO_4, 25 mM sodium bicarbonate, 25 mM sucrose, 1.8 mM $CaCl_2$, 0.04% phenol red, pH 7.3), inside an anaerobic chamber with a mixture of 5% CO_2, 7.5% H_2 and 87.5% N_2 (OGD chamber, Thermo Forma 1029, Thermo Fisher Scientific, Waltham, MA), at 37ºC for 2h. Control neurons were placed in a similar buffer, containing 25 mM

glucose, instead of sucrose (sham medium) and kept for 2h in an oxygenated incubator. After the insult, neurons were placed in their conditioned medium and returned to the normoxic incubator for an additional period of recovery that lasted up to 24h.

When indicated, MK-801 or ifenprodil were added to the medium 15 min prior to stimulation and kept during the stimulation and post-stimulation periods, at the indicated concentrations. The cell-permeable peptides, L-TAT and L-TAT-NR2B$_{9c}$ were pre-incubated 1h prior to stimulation and kept for the remainder of the experiment.

Immunocytochemistry

Cortical mouse neurons were labeled with an anti-GFP antibody (1:500 - #598, MBL International, Woburn, MA), to detect transfected neurons. Briefly, following methanol fixation (10 min at -20°C), the coverslips were incubated with 10% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hour at 37°C to prevent nonspecific antibody binding, and incubated overnight with the primary antibody in 3% BSA at 4ºC. Secondary antibody anti-rabbit-Alexa 488 (Jackson ImmunoResearch, Suffolk, UK) was also prepared in 3% BSA and incubated at 37°C for 1 hour. The coverslips were extensively washed with PBS after antibody incubations and were then mounted in fluorescence mounting medium from DAKO (Glostrup, Denmark), after the staining with Hoechst 33342 in order to visualize cell nuclei.

Nuclear Morphology

Neuronal death was assessed 24h following the OGD insult by analysis of the nuclear morphology. For that purpose, fixed neurons were stained with the nuclear dye Hoechst 33342 (2 µg/ml) for 15 min at RT and then mounted in florescence mounting medium. Neurons were analyzed under fluorescence microscopy for chromatin condensation, a feature of apoptotic cells. At least 200 cells were counted in each coverslip. When using transfected cells, GFP-positive neurons were detected using the green fluorescence filter and nuclear morphology was analyzed using the blue fluorescence filter. The totality of transfected cells in each coverslip was counted. The results were expressed as the percentage of cell death (dead cells/total number of cells).

LDH release assay

At 24h of recovery after the OGD challenge, the lactate dehydrogenase (LDH) release to the extracellular medium was evaluated by a colorimetric assay using the CytoTox96 Non-Radioactive assay kit (Promega, Madison, WI), according to the manufacturer's instructions. The percentage of LDH release is a measure of the extent of neuronal death and was determined as the ratio between LDH activity in the extracellular medium and total LDH activity obtained after complete cell lysis with Triton X-100. All experiments were carried out in duplicate or triplicate, for each independent experiment, as previously described (Vieira et al., 2010).

Statistical Analysis

Results are presented as means ± S.E.M. of the indicated number of experiments. Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by the Dunnett's or the Bonferroni's test. These statistical analyses were performed using the software package GraphPad Prism 5.

Results

The GluN2B Subunit is Determinant for OGD-induced Neuronal Death

Excitotoxicity is one of the major hallmarks of brain ischemic injury, as a consequence of excessive glutamate release to the synapse and dysfunction of glutamate buffering mechanisms (Olney, 1969; Lipton and Rosenberg, 1994; Wang and Qin, 2010), with resultant Ca^{2+} entry in neurons via GluRs (Arundine and Tymianski, 2003; Forder and Tymianski, 2009). Evidence in the literature suggests that both NMDAR (Hardingham et al., 2002; Arundine and Tymianski, 2004) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPARs), specifically Ca^{2+}-permeable (CP)-AMPARs (Gorter et al., 1997; Pellegrini-Giampietro et al., 1997) are responsible for the excitotoxic component of ischemic insults. In this study we used OGD in hippocampal and cortical cultured neurons as an *in vitro* model of global ischemia. To validate this model, we verified whether it induced an excitotoxic component of neuronal death in primary cultures of hippocampal neurons. For that purpose, we deprived hippocampal neurons from both oxygen and glucose for 2h and

then placed them back in glucose-containing and oxygenated conditions for an additional period of 24h. To address the role of NMDARs in this system we added NMDAR antagonists 15 min prior to OGD stimulation and kept the compounds for the remainder of the experiment (Fig 2.1-A). Using the LDH activity assay, we observed that the NMDAR antagonist MK-801 (10 μM) was effective in reducing neuronal death induced by OGD (from 37.1±4.5% to 19.7±2.9% of LDH release). Interestingly, we also observed significant neuroprotection by the selective GluN2B antagonist ifenprodil (3 μM reduced LDH release to 20.5%±5.3). This suggests that receptors containing this subunit in their composition are responsible, at least partially, for the neuronal death induced by excitotoxicity in this system. These results demonstrate the occurrence of a NMDAR-mediated excitotoxic component in the OGD model.

The neuroprotective effect of ifenprodil (Fig 2.1-A), along with the evidence in the literature of a relevant role of the GluN2B subunit in excitotoxic events (Kapin et al., 1999; Aarts et al., 2002), prompted us to investigate the actual role of GluN2B under OGD conditions. Thus, we took advantage of a GluN2B$^{-/-}$ system to address this issue. To confirm that the GluN2B subunit is determinant for the excitotoxic component of OGD-induced damage, we compared the neuronal death elicited by OGD in GluN2B$^{-/-}$ neurons and in WT neurons (Fig 2.1-B, C). In WT mouse cortical neurons, OGD induced cell death (35.12±3.4%), as determined by analyzing the nuclear morphology. On the contrary, the extent of cell death in GluN2B$^{-/-}$ neurons was much restricted in this assay and OGD-elicited death was not significantly different from basal neuronal death (Fig 2.1-B). This result suggests that, in the absence of GluN2B expression, neurons become more resistant to ischemic insults, similarly to what has been observed for NMDA-induced excitotoxic neuronal damage (Liu et al., 2007).

To confirm that this increased resistance is a result of the absence of GluN2B excitotoxic signaling, and not only an effect of reduced levels of NMDARs at the surface, we reintroduced the GluN2B subunit in cortical neuronal cultures and analyzed cell death elicited by OGD in transfected cells. We observed that expression of GluN2B rescues the toxic effect of the OGD challenge (from 11.1% to 33.0% of cell death) (Fig 2.2-C). The GluN2B and GluN2A subunits present low homology at the C-terminal intracellular domain (Fig. 2.2.-A), and their differential role in cell death mechanisms could be a consequence of C-terminal-mediated processes. To test this idea we introduced a chimeric construct comprising YFP-GluN2B with the GluN2B C-terminal domain swapped by that of the GluN2A subunit (Fig. 2.2-B). In fact, introduction of this construct in GluN2B$^{-/-}$ neurons failed to rescue the neuronal death that we observed for the WT GluN2B subunit (5.7% of neuronal death) (Fig 2.2-C). Importantly, this chimeric

subunit is still targeted to the membrane (Ann Marie Craig personal information, data not shown), further supporting the notion that the WT GluN2B subunit expression is determinant for NMDAR-dependent toxicity and that the C-terminal domain of this subunit is crucial for induction of excitotoxic mechanisms. This is in accordance with a previous report where similar chimeric subunits were used, that demonstrated that these subunits did not alter NMDA currents and the proportion of extrasynaptic receptors, suggesting that these subunits form functional NMDARs that are trafficked to the cell membrane.

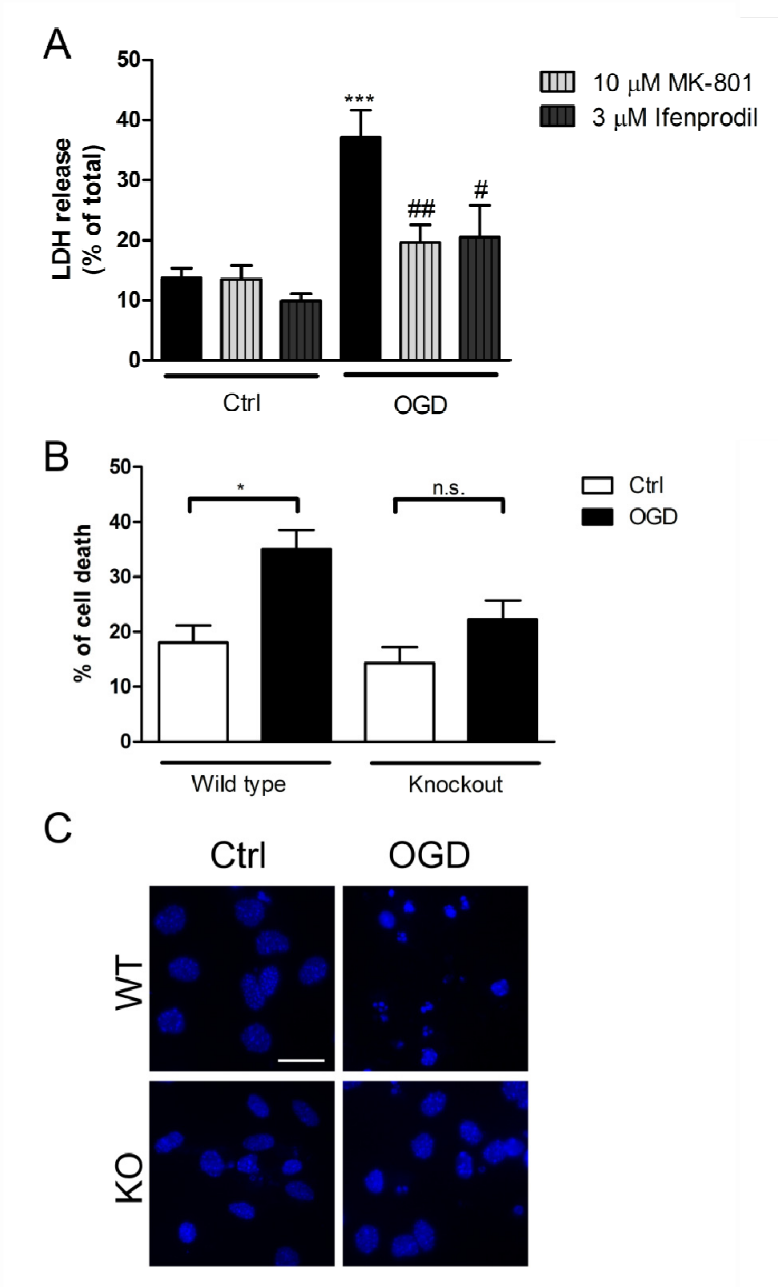


Figure 2.1. OGD induces excitotoxic neuronal death that is prevented in GluN2B$^{-/-}$ neurons. (A) Mature cultures of hippocampal neurons (15 DIV) were submitted to OGD.

For that purpose, neurons were placed in a glucose–free saline buffer and deprived of oxygen for 2h. Control neurons were placed in a similar saline buffer containing glucose and kept in an air/CO_2 chamber for the same period of time. The cultures were then transferred to the conditioned medium and returned to the air/CO_2 incubator for 24h. The NMDAR antagonist – MK-801: 10 μM, n=7; and the GluN2B antagonist – ifenprodil: 3 μM, n=4 were added 15 min prior to stimulation and kept during the stimulation and post-stimulation periods. The LDH activity was assayed as indicated in the manufacturer's protocol. Bars represent the mean ± SEM of the indicated number of experiments. *Significantly different from control (***p<0.001 (compared to Control); $^{\#}$p<0.05, $^{\#\#}$p<0.01 (compared to OGD), One way ANOVA followed by Bonferroni's multiple comparison test). **(B)** Mature cultures of WT or GluN2B$^{-/-}$ mouse cortical neurons (15 DIV) were stimulated as rat hippocampal neurons, and 24h after the insult nuclear morphology of cortical neurons was analyzed. The results were expressed as the percentage of dead cells relatively to the total number of cells. Bars represent the mean ± SEM of 4 independent experiments. *Significantly different from control (*p<0.05, Student's *t* test). **(C)** Representative images from B. Scale bar – 20 μm.

C-terminal-specific Interactions are Determinant for GluN2B-mediated Toxicity

Recently, a specific role of the C-terminal domains of GluN2B and GluN2A was suggested to determine the activation of pro-death or pro-survival signaling pathways, respectively (Martel et al., 2012). This is further supported by the fact that the C-terminus is the most divergent region between GluN2A and GluN2B subunits (Fig 2.2-A). Thus, we studied the C-terminal domain of GluN2B, using diverse mutant forms of the subunit (Fig 2.2-B), to identify molecular determinants required for cell death. In order to identify relevant interactions established between the C-terminal domain of the GluN2B subunit and intracellular proteins that are relevant for mediating a toxic pathway, we transfected GluN2B$^{-/-}$ cortical neurons with C-terminus mutants of GluN2B subunits, for known interaction sites. Of note, all of the mutants used were targeted to the cell surface (Ann Marie Craig personal information, unpublished results), thus, the effects observed are specific to the mutation and not a consequence of reduced levels of NMDARs at the surface.

The PDZ-binding domain of GluN2B has been shown to be important for activation of excitotoxic mechanisms (Sattler et al., 1999; Aarts et al., 2002; Cao et al., 2005). This domain was reported to be involved in the interaction with PSD95 which, upon NMDAR overactivation, leads to the recruitment of the Ca^{2+}-dependent neuronal nitric oxide synthase (nNOS) to the vicinity of NMDARs, thereby contributing to neuronal death

through nitric oxide (NO) production (Aarts et al., 2002; Zhou et al., 2010). Accordingly, we found that expression of the GluN2B subunit with a mutation in the PDZ-binding domain in GluN2B$^{-/-}$ neurons fails to rescue OGD toxicity (Fig 2.3-A). Furthermore, to determine whether we could reproduce the neuroprotective effect of disrupting the coupling between GluN2B and PSD95, we used a cell permeable peptide (L-TAT-NR2B$_{9c}$), which targets this interaction (Aarts et al., 2002). We observed that this peptide significantly protected rat hippocampal neurons from the OGD-induced death (Fig 2.3-C).

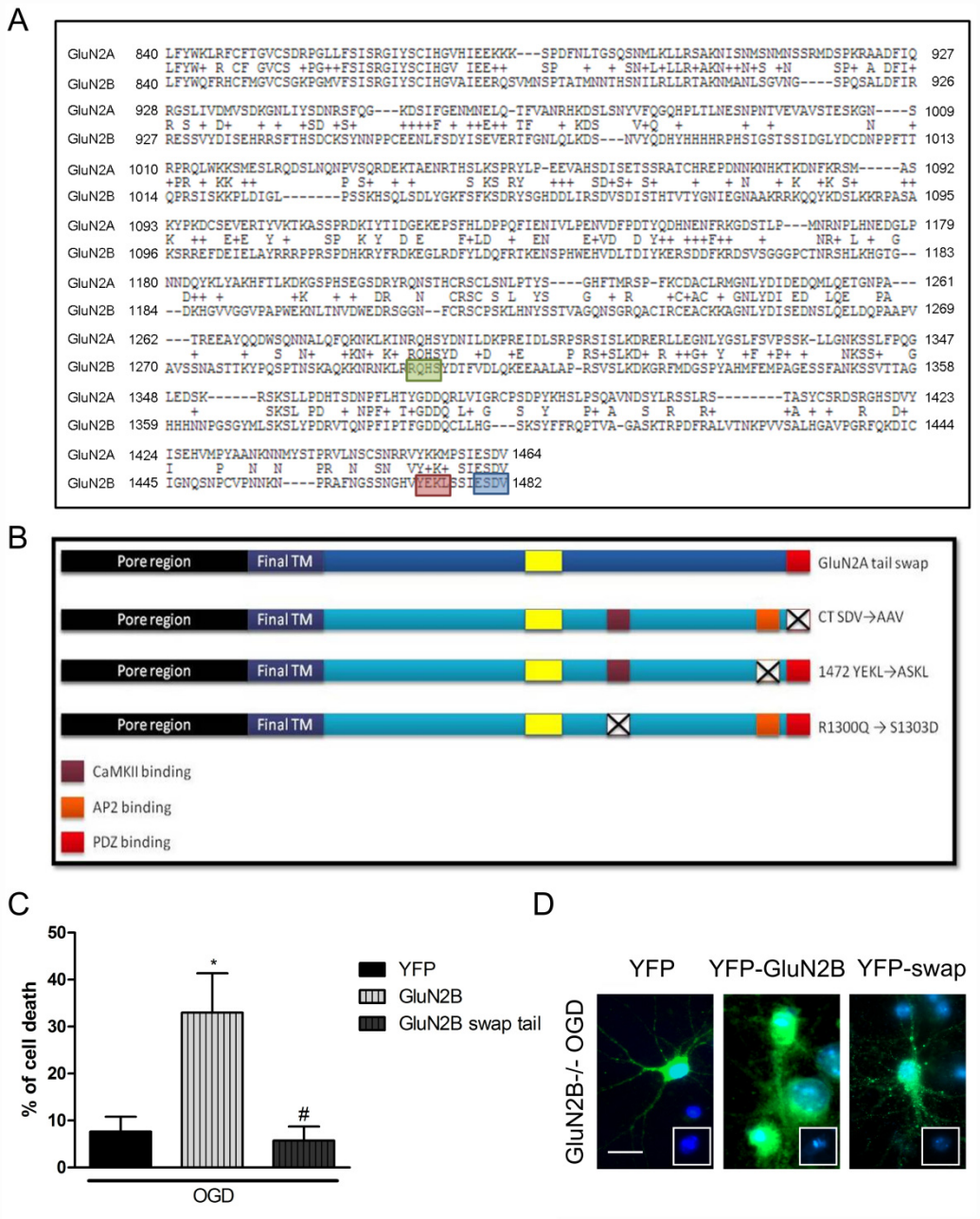


Figure 2.2. The C-terminal domain of the GluN2B subunit determines its toxic effect following OGD. (A) Alignment of the C-terminal domains of the mouse GluN2A

(NP_032196) and GluN2B (NP_032197) subunits. There is a high level of divergence between the C-terminus of GluN2A and GluN2B, which may contribute to their differential roles in excitotoxic mechanisms. **(B)** Diagram of the different mutant GluN2B subunits used in this work. A mutant consisting of a substitution of the C-terminal domain for that of the GluN2A subunit (swap tail) was developed. Additionally, the C-terminal domain of the GluN2B subunit was mutated in different domains, to produce an AP2 binding mutant (YEKL → ASKL), a CaMKII binding mutant (RQHS → QQHD) and a PDZ domain mutant (ESDV → EAAV). **(C)** Reintroduction of the GluN2B subunit in GluN2B$^{-/-}$ neurons rescues OGD-induced toxicity. Cortical neurons from GluN2B$^{-/-}$ animals were electroporated (5 µg/1500000 cells) with constructs encoding the YFP tag (n=4), the YFP-GluN2B subunit (n=4) or the YFP-GluN2B-swap tail (n=3) using the AMAXA system. After 15 DIV, the cultures were submitted to OGD. Twenty-four hours after the insult, cells were fixed with methanol. The nuclear morphology of transfected cells was analyzed and expressed as the number of dead (apoptotic-like cells) relative to the total number of transfected cells. (*$p<0.05$, relative to OGD-YFP; $^{\#}p<0.05$, relative to OGD-GluN2B, One way ANOVA followed by Bonferroni's multiple comparison test). **(D)** Representative images from C. Scale bar – 20 µm; insert – 10 µm. Inserts show nuclear morphology of a transfected cell.

The PDZ-binding domain of GluN2B has been shown to be important for activation of excitotoxic mechanisms (Sattler et al., 1999; Aarts et al., 2002; Cao et al., 2005). This domain was reported to be involved in the interaction with PSD95 which, upon NMDAR overactivation, leads to the recruitment of the Ca^{2+}-dependent neuronal nitric oxide synthase (nNOS) to the vicinity of NMDARs, thereby contributing to neuronal death through nitric oxide (NO) production (Aarts et al., 2002; Zhou et al., 2010). Accordingly, we found that expression of the GluN2B subunit with a mutation in the PDZ-binding domain in GluN2B$^{-/-}$ neurons fails to rescue OGD toxicity (Fig 2.3-A). Furthermore, to determine whether we could reproduce the neuroprotective effect of disrupting the coupling between GluN2B and PSD95, we used a cell permeable peptide (L-TAT-NR2B$_{9c}$), which targets this interaction (Aarts et al., 2002). We observed that this peptide significantly protected rat hippocampal neurons from the OGD-induced death (Fig 2.3-C).

To address a putative role of other known domains of the C-terminus of GluN2B, we expressed two additional GluN2B mutants in the GluN2B$^{-/-}$ neurons. Both mutants – one for the CaMKII binding site and the other for the AP2 interaction domain – were unable to rescue OGD-induced toxicity (Fig 2.4-A). These results demonstrate that GluN2B subunits induce neuronal death by a variety of mechanisms and

pathways, not only through Ca^{2+} influx, but also by activating diverse intracellular signaling pathways that have deleterious effects.

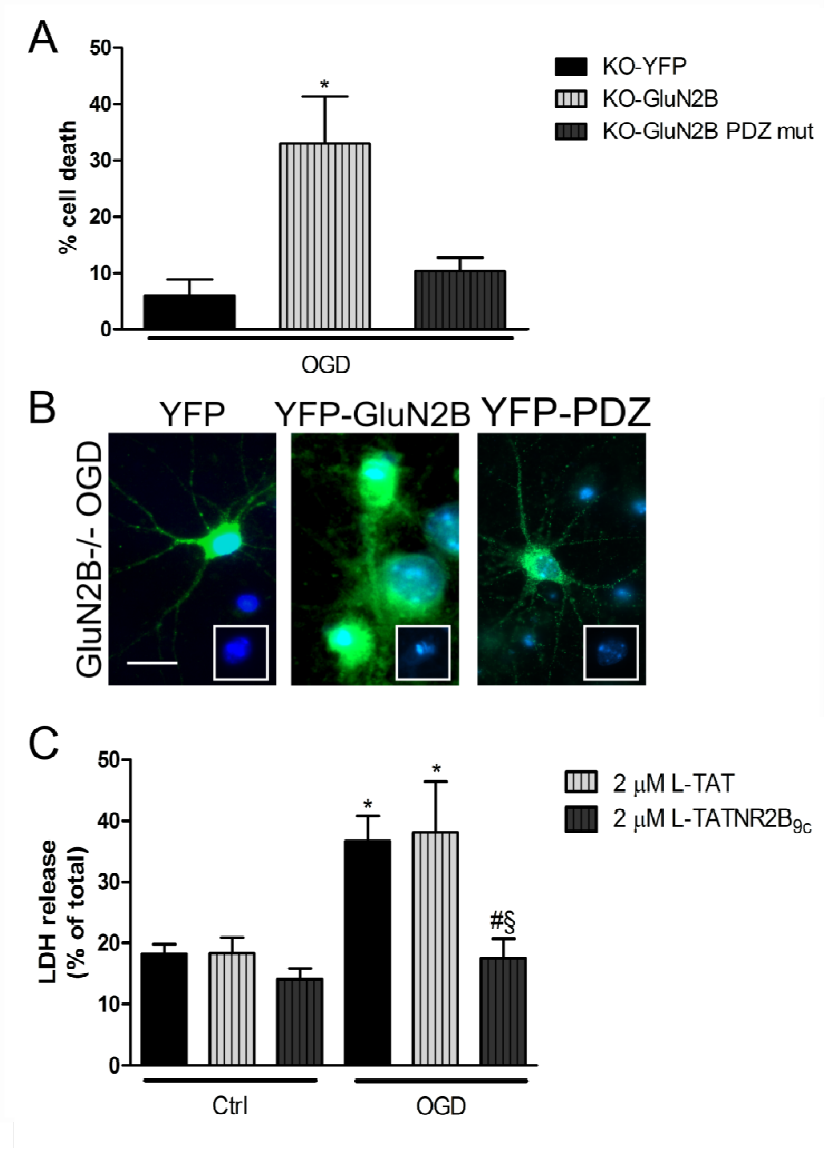


Figure 2.3. The PDZ-binding domain of the C-terminus of the GluN2B subunit is determinant for the OGD-induced neuronal death. For the OGD challenge, mature cultures of GluN2B$^{-/-}$ mouse cortical neurons (15 DIV) transfected with the indicated constructs or untransfected rat hippocampal neurons were placed in a glucose–free saline buffer and deprived of oxygen for 2h. Control neurons were placed in a similar saline buffer containing glucose and kept in an air/CO_2 chamber for the same period of time. The cultures were then transferred to the conditioned medium and returned to the air/CO_2 incubator for 24h. **(A)** After fixation with methanol, mouse cortical neurons were probed with an anti-GFP antibody to identify transfected neurons and with Hoechst 33342 in order to analyze the nuclear morphology of transfected neurons. The results were expressed as the percentage of dead cells relatively to the total number of transfected

cells. Bars represent the mean ± SEM of the indicated number of experiments (YFP – n=4; YFP-GluN2B – n=3; YFP-GluN2B-PDZ mutant – n=2). *Significantly different from OGD-YFP (*p<0.05, One way ANOVA followed by Bonferroni's multiple comparison test). **(B)** Representative images from A. Scale bar – 20 μm; insert – 10 μm. Inserts show nuclear morphology of a transfected cell. **(C)** One hour prior to the OGD challenge, rat hippocampal neurons were treated with the cell-permeating peptides (control TAT and TAT-NR2B9c) which were kept throughout the experiment. Twenty four hours after OGD the LDH activity of the extracellular medium was assayed. Bars represent the mean ± SEM of 5 independent experiments. *Significantly different from control (*p<0.05, relative to control, $^{\#}$p<0.05, relative to OGD, §p<0.05, relative to OGD-TAT, One way ANOVA followed by Bonferroni's multiple comparison test).

Discussion

The determinant contribution of the GluN2B subunit to excitotoxic neuronal death has been widely studied. Using a GluN2B$^{-/-}$ system, we intended to clarify the role of this subunit and, in particular, of its C-terminal domain, to the neuronal death induced by OGD, an *in vitro* insult more similar to cerebral ischemia than the exposure to NMDA or other GluR agonists. We observed that OGD-induced cell death is dependent on the expression of the GluN2B subunit, since it is abolished in GluN2B$^{-/-}$ neurons. Expression of YFP-GluN2B in these cultures promoted a component of excitotoxicity upon the OGD challenge while the expression of a chimera consisting of the N-terminus and transmembrane domains of GluN2B attached to the C-terminal domain of GluN2A was not capable of restoring cortical neuronal death in the GluN2B$^{-/-}$ cultures. Interestingly, we observed that several domains in the C-terminus of GluN2B are responsible for its function in excitotoxic phenomena. Indeed, we observed that not only is the PDZ-binding domain crucial for OGD-induced death, but also the interaction sites for both CaMKII and the clathrin adaptor protein AP2 contribute to the activation of cell death mechanisms in these conditions. Thus, this report provides new mechanisms of GluN2B-mediated excitotoxicity following *in vitro* ischemia.

OGD insults elicit excitotoxic cell death dependent on NMDA and AMPA/kainate receptors (KARs) (Goldberg and Choi, 1993; Gwag et al., 1995). This *in vitro* ischemic challenge induces long-lasting potentiation of NMDA responses in the vulnerable CA1 region of the hippocampus, while transiently depressing these responses in the more resistant CA3 area (Gee et al., 2006). In this report we confirm the role of NMDARs to

the damaging effect of the OGD challenge, by showing the neuroprotective effect of NMDAR antagonists.

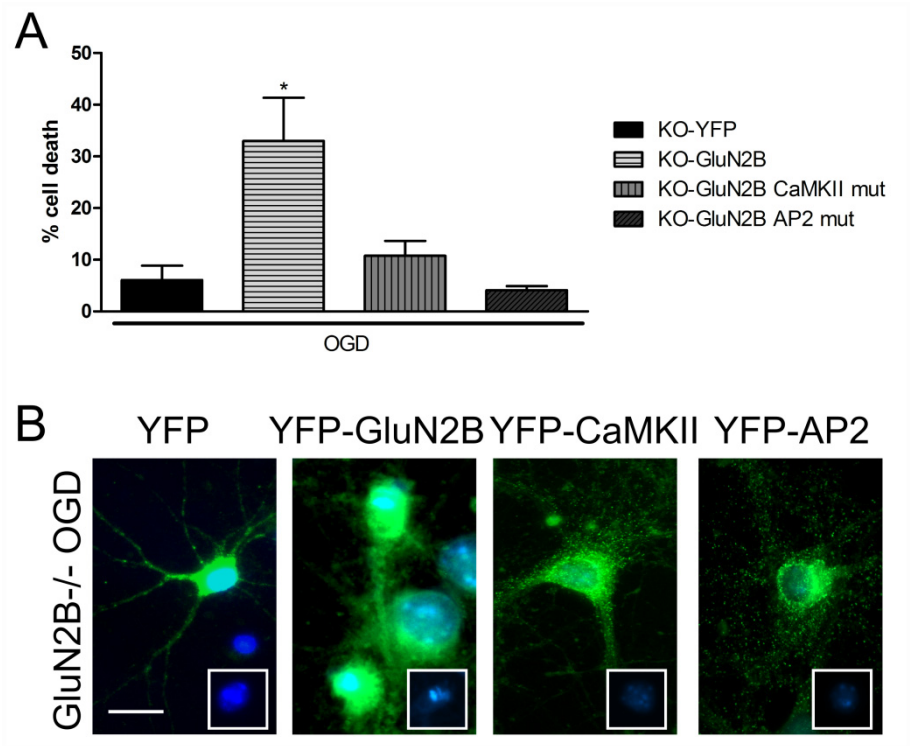


Figure 2.4. Several domains in the C-terminus of GluN2B are responsible for the subunit-specific toxicity. For the OGD challenge, mature cultures of GluN2B$^{-/-}$ mouse cortical neurons (15 DIV) transfected with the indicated constructs were placed in a glucose–free saline buffer and deprived of oxygen for 2h. Control neurons were placed in a similar saline buffer containing glucose and kept in an air/CO_2 chamber for the same period of time. The cultures were then transferred to the conditioned medium and returned to the air/CO_2 incubator for 24h. After fixation with methanol, neurons were probed with an anti-GFP antibody to identify transfected neurons and with Hoechst 33342 in order to analyze the nuclear morphology of transfected neurons. **(A)** The results were expressed as the percentage of dead cells relatively to the total number of transfected cells. Bars represent the mean ± SEM of the indicated number of experiments (YFP – n=4; YFP-GluN2B – n=3; YFP-GluN2B-CaMKII mutant – n=2; YFPGluN2B-AP2 mutant – n=2). *Significantly different from control (*$p<0.05$, One way ANOVA followed Dunnett's multiple comparison test). **(B)** Representative images from A. Scale bar – 20 µm; insert – 10 µm. Inserts show nuclear morphology of a transfected cell.

Synaptic and extrasynaptic NMDARs have been shown to be differentially regulated (Li et al., 2002) and to have opposing contribution to the neuronal death associated with excitotoxic phenomena (Hardingham et al., 2002; Hardingham and

Bading, 2010). Synaptic NMDAR activation leads to increased activity of phosphoinositide 3-kinase (PI3K)/Akt, phosphorylation of extracellular-signal regulated kinase (ERK) and transcriptional activity of cAMP-response-element-binding protein (CREB), which contributes to neuronal survival (Chandler et al., 2001; Hardingham et al., 2002; Papadia et al., 2005; Zhang et al., 2011). On the contrary extrasynaptic NMDARs are thought to contribute to activation of deleterious signaling pathways, such as p38 mitogen-activated protein kinase (MAPK), and to induce CREB shut-off, thereby promoting neuronal demise (Hardingham et al., 2002; Ivanov et al., 2006; Leveille et al., 2008; Wahl et al., 2009; Xu et al., 2009).

Interestingly, the GluN2A and GluN2B subunits of NMDARs have also been shown to exert opposing effects against excitotoxic insults (Wyllie et al., 2013). Indeed, while GluN2B antagonism or knock-down (KD) prevents neuronal death induced by *in vitro* and *in vivo* insults, GluN2A inhibition does not display any neuroprotective effect. In fact, selective activation of GluN2A-containing receptors has a prosurvival effect (Liu et al., 2007). The role of GluN2A subunits in survival signaling is further supported by reports showing its involvement in ischemic preconditioning phenomena, leading to CREB phosphorylation and transcriptional activity (Terasaki et al., 2010).

This prosurvival role of the GluN2A subunit has been challenged in several reports, demonstrating a lack of specificity of GluN2A inhibitors (Weitlauf et al., 2005; Frizelle et al., 2006; Neyton and Paoletti, 2006; Stanika et al., 2009), and in some reports pointing out a protective effect of interfering with the interaction between GluN2A subunits with PSD95 and Src, contributing to decreased GluN2A phosphorylation and decreased activation of nNOS (Zhang et al., 2007; Wang et al., 2010). In these reports, however, the authors did not address a putative effect on the GluN2B subunit. Furthermore, recent evidence reported a role for both subunits in either the survival as well as the toxic function of NMDARs. Indeed, both subunits are shown to contribute to activation of the CREB and brain-derived neurotrophic factor (BDNF) survival signaling, as well as for the shut-off of these pathways, supporting the hypothesis that synaptic NMDARs activate prosurvival signaling whilst the extrasynaptic ones activate prodeath mechanisms. Interestingly, they found that inhibition or KD of GluN2B subunits was more effective in rescuing neurons from excitotoxicity induced by NMDA or OGD, which was more evident with increasing intensity of the insults (Zhou et al., 2013). These findings complicate the interpretation of NMDAR function, since both location and subunit composition seem to contribute to the opposing effects of NMDARs. Interestingly, although neither GluN2A nor GluN2B are exclusively present in either site, they were found to be most abundant in the

synapse or in extrasynaptic locations, respectively (Li et al., 1998; Tovar and Westbrook, 1999; Groc et al., 2006).

In this report we did not address the role of the GluN2A subunit directly. Using a KO culture system for the GluN2B subunit we determined that this subunit is crucial for the excitotoxic events induced by OGD. Indeed, we observed that antagonism or absence of this subunit abrogates neuronal death induced by OGD. Ifenprodil, which is considered a highly selective GluN2B antagonist (Williams, 1993; Karakas et al., 2011; Ogden and Traynelis, 2011) abrogated OGD-induced toxicity in rat hippocampal neurons and the results with this antagonist are further supported by the absence of OGD-induced toxicity, relatively to the WT, in GluN2B$^{-/-}$ mouse cerebral cortical neurons. We confirmed the specificity of this effect by rescuing the toxicity of OGD when we introduced the GluN2B subunit in the GluN2B$^{-/-}$ neurons.

Whilst sharing high degree of similarity in their extracellular and transmembrane domains (69%), the mouse NMDAR subunits GluN2A and GluN2B share only 29% of identity in their C-terminal domains [Fig. 2.2-A, (Ryan et al., 2008)]. This suggests that the C-terminal domains of these two subunits are the main determinants of their specific functions in the cells, and their proposed differential role under excitotoxic conditions (Wyllie et al., 2013). Interestingly, a recent report by Martel and colleagues supports this notion. They describe that the C-terminal domain of GluN2B is the region of the subunit responsible for mediating its toxic effect. Indeed, when they used chimeric subunits, comprising the GluN2B subunit attached to the C-terminus of GluN2A, they failed to induce neuronal death following excitotoxic treatment with NMDA (Martel et al., 2012). It should be noted that the tail swapping does not seem to alter the gating properties of the receptors (Maki et al., 2012; Punnakkal et al., 2012), nor their targeting to the cell membrane (Ann Marie Craig personal communication, unpublished results) conferring specificity to the findings with this type of chimeric subunit. In this report we describe a comparable finding. Using a similar chimeric subunit, we observed that GluN2B swap tail failed to rescue the toxicity induced by OGD, unlike the WT GluN2B subunit. Importantly, not only does this result support a prominent role of the GluN2B subunit to the excitotoxic mechanisms, in detriment of the GluN2A subunit, specifically of its C-terminal domain, we also demonstrate the relevance of this effect by using an insult that more closely models *in vivo* ischemia.

One of the suggested mechanisms regulating the specific neurotoxic effect of GluN2B is the interaction between this subunit with PSD95, through its PDZ domain. The scaffold PSD95 couples to nNOS upon activation of NMDARs, contributing to

deleterious production of NO and mounting oxidative and nitrosative stress, as well as p38 activation (Sattler et al., 1999; Aarts et al., 2002; Cao et al., 2005; Zhou et al., 2010). To prevent the toxic effects of GluN2B-containing NMDARs, a peptide targeting the interaction between GluN2B and PSD95 (TAT-NR2B$_{9c}$) has been developed, in order to uncouple NMDARs containing the GluN2B subunit from nNOS activation. Several reports have shown a significant neuroprotective effect when disrupting this interaction (Aarts et al., 2002; Cui et al., 2007; Soriano et al., 2008; Martel et al., 2009a; Cook et al., 2012), which has the advantage of not interfering with the PSD apparatus. Similar strategies using cell permeable peptides targeting specific protein interactions have been adopted and were found to be similarly neuroprotective (Tu et al., 2010; Li et al., 2013). In this report we also demonstrate a neuroprotective effect of the peptide TAT-NR2B$_{9c}$, which is in accordance with these previous reports. Specifically, we demonstrate the relevance of the PDZ-binding domain by introducing a PDZ-binding domain mutant subunit, which is unable to bind PSD95 and fails to induce toxicity following OGD, as observed for the C-terminal domain chimera, relatively to the WT subunit. Again, our results gain functional significance due to the type of insult chosen, which correlates better with *in vivo* cerebral ischemia, while most reports use only excitotoxic insults.

Although the role of the PDZ-binding domain of the GluN2B subunit, and the interaction with PSD95, have been extensively associated with excitotoxic phenomena, a role for the remainder of the C-terminal domain of the subunit cannot be excluded in the neuronal demise that ensues in these conditions. However, to date, these mechanisms are still not completely elucidated. One possible additional mechanism of regulation of excitotoxicity is the phosphorylation of the subunit by CaMKII. The interaction between GluN2B and CaMKII was shown to be important for neuronal development and function (Wang et al., 2011). This kinase phosphorylates GluN2B at Ser1303 and this phosphorylation was found to regulate the interaction between CaMKII and GluN2B (Raveendran et al., 2009; O'Leary et al., 2011). The OGD challenge induces increased CaMKIIα autophosphorylation (Thr286) and CaMKIIα-dependent phosphorylation of GluN2B subunits. Phosphorylation of this residue causes increased Ca^{2+} influx, thereby contributing to neuronal demise. Moreover, GluN2B activation contributes to CREB shut-off, which is prevented by CaMKII inhibition (Farinelli et al., 2012). In this report we describe, for the first time, the abrogation of neuronal death induced by an ischemic stimulus elicited by a GluN2B mutant for the CaMKII binding site. Unlike the WT GluN2B, this mutant subunit failed to elicit neuronal death following the OGD challenge. This suggests that the interaction between GluN2B

and CaMKII is a determinant event in the extent of toxicity induced by *in vitro* ischemia. This mechanism may account for the differences observed between GluN2A and GluN2B subunits, since CaMKII was shown to bind with higher affinity to GluN2B subunits (Strack and Colbran, 1998).

Synaptic NMDAR localization has been shown to be regulated by tyrosine phosphorylation of the C-terminus of GluN2 subunits, which inhibits the binding of the clathrin adaptor protein AP2 (Roche et al., 2001; Vissel et al., 2001). The endocytosis of synaptic NMDARs seems to be differentially regulated from that of extrasynaptic NMDARs (Li et al., 2002). Interestingly, the PDZ-binding domain of the GluN2B subunit was shown to be also important for this regulation, counteracting the internalization of NMDARs (Roche et al., 2001; Prybylowski et al., 2005). Thus, an increase in the number of synaptic NMDARs was observed upon mutation of the AP2 binding site (Tyr1472) on GluN2B subunits (Prybylowski et al., 2005). We found a curious effect of the AP2-binding site mutant (YEKL). We observed that this mutant GluN2B subunit failed to rescue the damaging effect of the OGD challenge in GluN2B$^{-/-}$ neurons. This result may imply that, in the KO background used in this work, mutations in the AP2 binding site may target most receptors to synaptic sites, where they will most likely participate in prosurvival signaling, as suggested by several reports (Papadia et al., 2008; Al-Mubarak et al., 2009; Martel et al., 2009b). If this is the case, this evidence supports the notion of an intricate interplay between receptor subunit composition and localization in the prosurvival versus prodeath function of NMDARs. However, it should be noted that the AP2-binding site is in close proximity to the PDZ-binding domain, thus we cannot exclude a possible interference of the mutation of the former site with binding to the latter. This, however, seems unlikely, as mutants for the two distinct sites display differential effects on receptor localization and properties (Prybylowski et al., 2005).

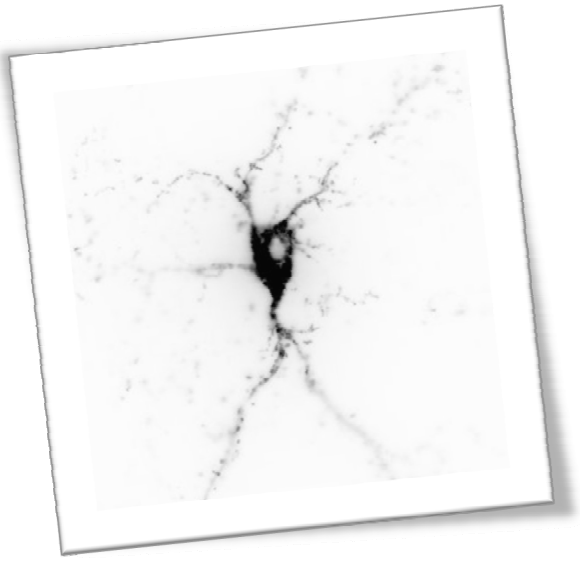
The several mechanisms of regulation of NMDAR-dependent toxicity described seem to have one common player, Ca^{2+}. Indeed, not only is the activation of nNOS (downstream of the interaction between GluN2B and PSD95) dependent on Ca^{2+} entry, but also CaMKII is activated by this cation. Additionally, a mechanism of regulation of NMDAR endocytosis during LTD has been demonstrated to be dependent on Ca^{2+}, by regulation via a Ca^{2+} sensor protein – hippocalcin. This protein binds to PSD95 and promotes dissociation of this protein from the receptor on sensing Ca^{2+}, thereby allowing the interaction of AP2 with NMDAR subunits, which promotes receptor internalization (Jo et al., 2010). Thus, upon overactivation of NMDARs, influx of Ca^{2+} may contribute to the internalization of synaptic NMDARs via a mechanism dependent

on AP2 and, simultaneously leads to activation of CaMKII and nNOs, favoring neuronal demise.

Overall, our results support the notion that the GluN2B subunit of NMDARs has a determinant role in the induction of toxicity, in ischemic conditions. Specifically, we provide evidence for the crucial role of its C-terminal domain for much of the toxicity of GluN2B, distinguishing it from the GluN2A subunit. Additionally, we also identified several mechanisms of regulation of this subunit, correlatable with GluN2B subunit toxicity. In conclusion, our results suggest that the toxicity mediated by GluN2B-containing NMDARs is the result of a complex interplay of factors, namely subunit phosphorylation, protein interactions and recycling. We identify, for the first time, a mechanism of regulation by the clathrin adaptor protein AP2 which may contribute to toxicity by promoting endocytosis of synaptic NMDARs.

Chapter III

Up-regulation of Endogenous RIP3 Contributes to Necroptotic Neuronal Death in Cerebral Ischemia



Abstract

Global cerebral ischemia induces selective neurodegeneration of the CA1 region of the hippocampus. The type of cell death that ensues may include different programmed cell death mechanisms namely apoptosis and necroptosis, a recently described type of programmed necrosis. We investigated whether necroptosis contributes to hippocampal neuronal death following oxygen-glucose deprivation (OGD), an *in vitro* model of global ischemia. We observed that OGD induced a death receptor (DR)-dependent component of necroptotic cell death in primary cultures of hippocampal neurons. Additionally, we found that this ischemic challenge upregulated the receptor-interacting protein kinase 3 (RIP3) mRNA and protein levels, with a concomitant increase of the RIP1 protein. Together, these two related proteins form the necrosome, the complex responsible for induction of necroptotic cell death. Interestingly, we found that caspase-8 mRNA, a known negative regulator of necroptosis, was transiently decreased following OGD. Importantly, we observed that the OGD-induced increase in the RIP3 protein was paralleled in an *in vivo* model of transient global cerebral ischemia, specifically in the CA1 area of the hippocampus. Moreover, we show that the induction of endogenous RIP3 protein levels influenced neuronal toxicity since we found that RIP3 knock-down (KD) abrogated the component of OGD-induced necrotic neuronal death while RIP3 overexpression exacerbated neuronal death following OGD. Overexpression of RIP1 also had deleterious effects following the OGD challenge. Taken together, our results highlight that cerebral ischemia activates transcriptional changes that lead to an increase in the endogenous RIP3 protein level which might contribute to the formation of the necrosome complex and to the subsequent component of necroptotic neuronal death that follows ischemic injury.

Introduction

The brain damage due to cerebral ischemia is one of the major causes of disability in the western world. Transient global cerebral ischemia that results from a lack of blood supply to the whole brain in consequence of cardiac arrest, leads to the selective and delayed death of certain populations of neurons. The hippocampus is one of the most severely affected areas in patients (Petito et al., 1987) and also in animal models of global ischemia (Kirino, 1982; Zukin et al., 2004). Global cerebral ischemic

insults can be simulated *in vitro* by performing oxygen-glucose deprivation (OGD) on primary neuronal cultures or slices, typically from the hippocampus or cortex (Goldberg and Choi, 1993; Martin et al., 1994; Calderone et al., 2003).

Cerebral ischemic insults both *in vivo* and *in vitro* induce necrotic as well as apoptotic neuronal death (Gwag et al., 1995; Martinez-Sanchez et al., 2004; Malagelada et al., 2005). In recent years, however, a novel type of cell death, called necroptosis, has been shown to contribute to ischemic injury (Degterev et al., 2005; Xu et al., 2010; Meloni et al., 2011; Northington et al., 2011). This type of regulated necrotic cell death was described to occur as a consequence of death receptor (DR) signaling, in conditions where apoptosis is inhibited or downregulated (Fiers et al., 1995; Vercammen et al., 1998; Van Herreweghe et al., 2010; Han et al., 2011). Although the necrotic component of cell death was so far considered to be unregulated and thus irreversible, a complete understanding of the mechanisms of regulated necrosis might provide new targets for the therapy of cerebral ischemia.

Tumor necrosis factor receptor 1 (TNFR1) signaling is complex and might have distinct outcomes. Upon tumor necrosis factor α (TNFα) binding to TNFR1, the receptor becomes activated and recruits a complex of proteins (complex I) to its vicinity that comprises the receptor-interacting protein kinase 1 (RIP1) and the TNFR associated factor 2 (TRAF2). This leads to nuclear factor kappa B (NF-κB) activation followed by expression of anti-apoptotic proteins, such as cellular inhibitor of apoptosis proteins (cIAPs) (Micheau and Tschopp, 2003), among others. The NF-κB activation downstream TNFR1 signaling is regulated by the post-translational modifications of RIP1 and TRAF2. When RIP1 is deubiquitinated by cylindromatosis (CYLD) or cIAP proteins are inhibited, the proteins of complex I dissociate from the receptor allowing the association, in the cytoplasm, of the so-called complex II or death inducing signaling complex (DISC), that recruits proteins such as Fas-associated protein with death domain (FADD), procaspase-8 and RIP1 (Micheau and Tschopp, 2003). Caspase-8 then becomes active and initiates the extrinsic apoptotic pathway. In cells with high expression of RIP3, this kinase might enter complex II due to the interaction with RIP1. Caspase-8 acts as a negative regulator of necroptosis, by promoting cleavage of RIP1 and RIP3 in complex II (Feng et al., 2007; Cho et al., 2009). In conditions where apoptosis is inhibited, RIP1 and RIP3 are able to induce necroptosis, by forming the complex IIb, or necrosome (Vercammen et al., 1998; Holler et al., 2000). While the precise mechanism by which RIP1 and RIP3 induce necroptosis is not fully understood it is known that their kinase activity is important for this process (Cho et al., 2009; He et al., 2009; Zhang et al., 2009) and recently it was shown that their

interaction is regulated by sirtuin2 (SIRT2)-dependent RIP1 deacetylation (Narayan et al., 2012). Nevertheless, the events that trigger the assembly of the necrosome in the context of cerebral ischemia are not yet known.

In this work we examined the neuroprotective effect of the necroptosis inhibitor necrostatin-1 (Nec-1) on OGD-challenged hippocampal neurons and we investigated the molecular determinants underlying OGD-induced necroptosis in hippocampal neurons, namely the role of RIP3 in this process. We show that ischemic insults induced an upregulation of the RIP3 mRNA and protein levels, accompanied by a transient caspase-8 mRNA downregulation. The changes in RIP3 protein level correlated with increased hippocampal neuronal death following OGD. Importantly, we also observed an increased RIP3 protein level in the CA1 region of the hippocampus of rats submitted to global cerebral ischemia *in vivo*. These results contribute to the elucidation of the mechanism of cerebral ischemia-induced necroptosis and therefore may pave the way to novel therapeutic targets for cerebral ischemia.

Materials & Methods

Materials

The antibodies against RIP1 (#610458) and RIP3 (#ab62344) were purchased from BD Biosciences (Franklin Lakes, NJ) and Abcam (Cambridge, MA), respectively. The antibody against α-Spectrin (MAB1622) was from Millipore (Billerica, MA) and the antibody against β-tubulin was purchased from Sigma-Aldrich (St. Louis, MO).

The neutralizing antibody against TNFα (NBP1-43235) was purchased from Novus Biologicals (Cambridge, UK). The necroptosis inhibitor Nec-1 (#036SC-200142) and its inactive analog (SC-204815) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the broad caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp(O-Me)-fluoromethyl ketone (zVAD.fmk, ab120382) was from Abcam. The N-methyl-D-aspartate receptor (NMDAR) antagonist (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801, #0924) was purchased from Tocris Bioscience (Bristol, UK).

The plasmids pBOB-mRIP3, encoding mouse RIP3, pBOB-mRIP1, encoding mouse RIP1, pBOB (empty vector) and the short hairpin RNA (shRNA) plasmids pLV-

H1-EF1a-puro-mRIP3-1465 (GCTCTCGTCTTCAACAACT) and pLV-H1-EF1a-puro-LacZ (control shRNA: GCAGTTATCTGGAAGATCA) were generous gifts from Duan-Wu Zhang and Jiahuai Han, Xiamen University, Xiamen, China. The plasmid pEGFP-N1-hRIP3 (#41387), encoding human RIP3 was obtained from Addgene (Cambridge, MA), provided by Francis Chan, University of Massachusetts (Worcester, MA) (Cho et al., 2009).

Cell Culture and Transfection

Primary cultures of hippocampal neurons were prepared from embryonic day 18 (E18) rats, as previously described (Santos and Duarte, 2008). Briefly, hippocampi were dissected and dissociated with trypsin (1.5 mg/ml) in Hank's Balanced Salt Solution (HBSS). Neurons were cultured in multiwell (MW) plates with or without glass coverslips, coated with poly-D-lysine (PDL), at a density of 85,000 cells/cm^2, in Neurobasal® medium (Gibco®, Life Technologies™, Paisley, UK) supplemented with 2% NeuroCult™ SM1 Neuronal Supplement (StemCell™ Technologies, Grenoble, France), 0.5 mM glutamine, 0.125 µg/ml gentamicin and 25 µM glutamate. Neuronal cultures were maintained at 37ºC in a humidified incubator with 5% CO_2 up to 15 days *in vitro* (DIV). At DIV 7, the cultures were fed with fresh supplemented neurobasal medium without glutamate.

For overexpression studies, neurons cultured in 12MW plates were co-transfected at DIV 7 using the calcium phosphate precipitation method (Dahm et al., 2008). Briefly, precipitates containing 1.5 µg of GFP and 3 µg of RIP3, RIP1 or pBOB (empty vector) plasmids were prepared using the following solutions: TE (1 mM Tris-HCl pH 7.3, 1 mM EDTA), 2x HEBS (12 mM Dextrose, 50 mM HEPES, 10 mM KCl, 280 mM NaCl and 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.2) and $CaCl_2$ (2.5M $CaCl_2$ in 10 mM HEPES, pH 7.2), and allowed to develop for 30 min. The precipitates were added to the neurons, in the presence of 2 mM kynurenic acid (Sigma-Aldrich, St. Louis, MO), and incubated for 3h at 37ºC. After the transfection period, the cells were washed with Neurobasal medium containing 2 mM kynurenic acid (HCl was added to keep an acidic pH to dissolve the precipitates) and then placed in conditioned medium until DIV 15.

For knock-down (KD) experiments, hippocampal cultures were prepared from E18 mice similarly to rat neuronal cultures, but papain was used for tissue dissociation (20 units/ml, 10 min, 37°C), instead of trypsin, as described (Ferreira et al., 2011). Hippocampal mouse neurons were maintained in supplemented Neurobasal Medium

(2% SM1, 0.5 mM Glutamine, 0.125 µg/ml gentamicin and 20 µg/ml insulin) at 37°C, in a humidified incubator with 5% CO_2. At DIV 7, mouse neurons (cultured in 24MW plates) were co-transfected as described for rat cultures, using 0.75 µg of GFP and 1.5 µg of the shRNAs for LacZ (control) or RIP3. For rescue experiments, 1.5 µg of hRIP3 were used instead of the GFP plasmid.

Oxygen-Glucose Deprivation (OGD) Challenge

At DIV 15, hippocampal neurons were challenged with an *in vitro* ischemic stimulus. For that purpose, neurons were placed in a glucose-free deoxygenated buffer medium (OGD medium – 10 mM HEPES, 116 mM NaCl, 5.4 mM KCl, 0.8 mM NaH_2PO_4, 25 mM sodium bicarbonate, 25 mM sucrose, 1.8 mM $CaCl_2$, 0.04% phenol red, pH 7.3), inside an anaerobic chamber with 5% CO_2 (OGD chamber, Thermo Forma 1029, Thermo Fisher Scientific, Waltham, MA), at 37ºC for 2h. Control neurons were placed in a similar buffer, containing 25 mM glucose, instead of sucrose (sham medium), and kept for 2h in a humidified incubator with 5% CO_2/95% air, at 37ºC. After incubation, the neurons were placed in their conditioned medium and returned to the normoxic incubator for a period of recovery that lasted up to 24h.

When indicated, chemical compounds such as receptor antagonists, Nec-1, zVAD.fmk or the TNFα neutralizing antibody were added to the medium 1h prior to stimulation and kept during the stimulation and post-stimulation periods, at the indicated concentrations.

RNA extraction

Total RNA from DIV 15 hippocampal neurons was extracted with TRIzol® Reagent (Invitrogen, Madrid, Spain), following the manufacturers' specifications. The total amount of RNA was quantified spectrophotometrically (Thermo Nanodrop 2000, Thermo Fisher Scientific, Wilmington, DE) and the RNA quality and integrity evaluated using the Experion automated gel electrophoresis system (BioRad, Hercules, CA).

qPCR

For cDNA synthesis, 1 µg of total RNA was used with the iScript™ cDNA Synthesis Kit (BioRad), according to the manufacturers' instructions. For quantitative real-time PCR (qRT-PCR) 20 µl reactions were prepared with 2 µl of 1:10 diluted cDNA, 10 µl of 2x iQ™ SYBR® Green Supermix (BioRad) and specific primers at 250 nM, as follows: β-Actin (*Actb*) forward – 5'-CGTCACCTACTCTAACCG-3', reverse – 5'-CTTGTGCTATCTGCTCATC-3'; *Caspase-8* forward – 5'-GGAACCAACTATGA TGAAGAG-3', reverse – 5'-GCAATCACTGAAGGACAC-3'; Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) forward – 5'- AACCTGCCAAGTATGATG-3', reverse – 5'-GGAGTTGCTGTTGAAGTC-3'; *Rip3* forward – 5'-TTCCAAGACTGTG AATCAA-3', reverse 5'-CACTGCTTCTGTACTGAG-3'.

The fluorescent signal was measured after each elongation step of the PCR reaction in the iQ5 Multicolor Real-Time PCR Detection System (BioRad), and was used to determine the threshold cycle (C_t) as previously described (Manadas et al., 2009). Melting curves were performed in order to detect non-specific amplification products, a non-template control was included in all assays, and for each set of primers a standard curve was performed to assess primer efficiency. Reactions were run in duplicate. The level of expression of each gene was analyzed at 7 and 24h of recovery following OGD and normalized to the level of expression of the gene in the corresponding control conditions. All C_t values were normalized relatively to two internal control genes, *Actb* and *Gapdh*, using the GenEx software (MultiD Analyses).

Nuclear Morphology

Neuronal death was assessed by analysis of the nuclear morphology 24h following the OGD insult. For that purpose, neurons were stained with the nuclear dyes propidium iodide (PI – 2 µg/ml) and Hoechst 33342 (2 µg/ml) for 15 min at 37ºC, and immediately analyzed under fluorescence microscopy for the incorporation of PI and chromatin condensation. The cellular membranes are impermeable to PI, thus this dye only penetrates the cells upon membrane rupture. Hence, PI is considered as a necrotic marker, since membrane rupture is one of the main features of necrotic cell death. When using a primary culture system, we should take into account that some cells that activate apoptosis undergo secondary necrosis. For that reason, we could not attribute PI uptake solely to primary necrosis. On the other hand, Hoechst 33342 is cell permeable, thus we analyzed the nuclear morphology and extent of chromatin

condensation and pyknosis to classify cells as apoptotic. For the necrotic-like neuronal death, we considered cells that are positive for PI staining, but in the absence of pyknosis. At least 500 cells were counted in each coverslip. The results were expressed as the percentage of necrotic cell death (PI positive without pyknosis/total number of cells) or percentage of apoptotic-like cell death (apoptotic-like nuclei/total number of cells). When using transfected cells, GFP-positive neurons were detected using the green fluorescence filter and PI incorporation confirmed using the red fluorescence filter. The totality of transfected cells in each coverslip was counted. The results were expressed as the percentage of necrotic cell death (PI positive/total number of transfected cells).

Lactate Dehydrogenase (LDH) Release Assay

At 24h of recovery after the OGD challenge, the LDH release to the extracellular medium was evaluated by a colorimetric assay using the CytoTox96 Non-Radioactive assay kit (Promega, Madison, WI), according to the manufacturer's instructions. The percentage of LDH release was determined as the ratio between LDH activity in the extracellular medium and total LDH activity obtained after complete cell lysis with Triton X-100. All experiments were carried out in duplicate or triplicate, for each independent experiment, as previously described (Vieira et al., 2010).

Protein extracts, SDS-PAGE and Western Blotting

Twenty four hours after OGD, cells were washed with ice-cold phosphate-buffered saline (PBS) and 1x sample buffer (125 mM Tris, pH 6.8, 2% SDS, 100 mM DTT, 20% glycerol and 0.005% bromophenol blue diluted in 50 mM HEPES, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 2 mM Na_3VO_4, 50 mM NaF, 1% Triton X-100, 10% glycerol, pH 7.4) was added. Cells were scraped, sonicated and denatured at 95ºC, for 10 min. Fifty µl of each protein extract were separated by SDS-PAGE, using 10% polyacrilamide gels, and then transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with Tris-buffered saline-Tween20 (TBS-T) (20 mM Tris, 137 mM NaCl, pH 7.6, 0.1% Tween20) with 5% non-fat milk, for 1h at room temperature, and then incubated with the primary antibody in TBS-T 5% milk, for 1h at room temperature. Alternatively, the incubation with the primary antibody was performed overnight at 4°C. After extensive washing, membranes were incubated with the secondary antibody conjugated with alkaline phosphatase for 1h at room

temperature. After additional washes, the membranes were developed using the enhanced chemifluorescence (ECF) substrate, and scanned on the Storm 860 Gel and Blot Imaging System (Amersham Biosciences, Buckinghamshire, UK). The density of the bands was analyzed with ImageQuant 5.0 software. For subsequent reprobing, the membranes were stripped of antibody with NaOH 0.2 M, blocked again and incubated with the appropriate antibodies.

Transient Global Cerebral Ischemia in the Rat

Experiments were carried out in accordance with the Guidelines of the European Union Council (86/609/EU), following Spanish regulations (RD 1201/2005, BOE 252/34367–91, 2005) for the use of laboratory animals, and were approved by the Scientific Committee of the University of León. All efforts were made to minimize animal suffering and to reduce the number of animals used. Global cerebral ischemia was performed as previously described (Montori et al., 2010), with slight modifications.

Eight 3-month-old Sprague-Dawley male rats weighing 350–450 g were housed under standard temperature (22 ± 1ºC) in a 12h light/dark controlled environment with free access to food and water. The animals were divided randomly into ischemic and sham groups. Rats were placed in the anesthesia induction box supplied with 4% isoflurane (IsoFlo, Abbott Laboratories Ltd) at 3 L/min in 100% oxygen. Animals were maintained under anesthesia with a flux of 1.5–2.5% isoflurane at 800 mL/min in 100% oxygen, delivered through a face mask. Both common carotid arteries were exposed through a midline incision and transient global ischemia was induced by bilateral common carotid artery occlusion for 15 min with atraumatic aneurysm clips and a moderate hypotension (40–50 mm Hg). Hypotension was carried out by exsanguination. The femoral artery was exposed and catheterized thus allowing continuous recording of arterial blood pressure. Heparin was supplied to the animal at 50 UI/kg through this catheter and 50 UI heparin were maintained in 3 ml saline in the syringe. Then, about 8 ml of blood were slowly extracted (1 ml/min) through the catheter and collected in the syringe until the desired hypotension was reached that was maintained introducing or extracting blood in the artery. Rectal temperature was controlled at 36 ± 1ºC during surgery with a feedback regulated heating pad. After ischemia, blood was returned to the animal at 1 ml/min until the animal arterial blood pressure recovered. The catheter was then removed and the animal was sutured. After regaining consciousness, animals were maintained in an air-conditioned room at 22 ±

1ºC during 48h. For sham-operated rats, all procedures were performed exactly as for ischemic animals with the exception that the carotid arteries were not clamped.

Two days after the ischemic insult, the animals were decapitated and their brains were rapidly removed. The CA1, the CA3, the dentate gyrus (DG) and the cerebral cortex (Ctx) were obtained using a dissecting microscope, frozen in liquid nitrogen and stored at -80ºC until use. On the day of the experiment, tissue sections were weighted and 1.5 mg of tissue from each area of the hippocampus and 5 mg from the cortex were homogenized with 125 µl (for the hippocampus) or 250 µl (for the cortex) of 1x sample buffer [125 mM Tris, pH 6.8, 2% SDS, 100 mM DTT, 20% glycerol and 0.005% bromophenol blue diluted in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton, 1 mM EDTA, 1 mM NaF, 10% glycerol, 150 mM NaOH and supplemented with protease inhibitors 1 mM phenylmethanesulfonyl fluoride (PMSF) and 1 µg/ml CLAP (chymostatin, leupeptin, antipain and pepstatin)]. Brain extracts were then sonicated and denatured at 95ºC for 5 min, and 50 µl of each sample were applied on SDS-PAGE.

Statistical Analysis

Results are presented as means ± S.E.M. of the indicated number of independent experiments. The normality of the data was assessed using the Kolmogorov-Smirnov test. Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by the Bonferroni's test. Alternatively, Student's *t*-test was applied on log-transformed data (for qPCR analysis and *in vivo* western blot analysis). These statistical analyses were performed using the software package GraphPad Prism 5.

Results

OGD Induces a Component of Necroptotic Neuronal Death

Emerging evidence suggests that necroptosis contributes to ischemic brain injury *in vivo* (Degterev et al., 2005; Xu et al., 2010; Northington et al., 2011). This evidence is mostly based on the neuroprotective effect of Nec-1, an inhibitor of necroptosis, against this type of insult. We investigated whether necroptotic neuronal

death occurs when hippocampal neurons are submitted to OGD, an *in vitro* model of global ischemia, more amenable to the molecular dissection of cell death mechanisms. The OGD challenge consists of combining the deprivation of both oxygen and glucose, thereby mimicking the lack of blood supply that occurs during ischemia. To study the putative contribution of necroptosis for the OGD-induced neuronal death, we incubated primary cultures of rat hippocampal neurons with Nec-1 or its inactive analog. Using different cell death assays we confirmed that Nec-1 had a neuroprotective effect against OGD-induced hippocampal neuronal death suggesting a component of necroptosis (Fig 3.1). In fact, using the nucleic acid dyes PI and Hoechst 33342, we observed that Nec-1 (20 μM) significantly reduced necrotic neuronal death from 12.4 ± 1.0% to 8.9 ± 0.4% (Fig 3.1-A) without having an effect on the apoptotic-like neuronal death component (30.4 ± 6.2% cell death on the OGD condition and 32.7 ± 4.7% with Nec-1) (Fig 3.1-C). We performed this analysis by counting the number of PI positive nuclei that do not present pyknosis, which correspond to necrotic cells, while the apoptotic-like cells presented chromatin condensation with Hoechst staining. When we used the LDH assay, which is an indirect measure of membrane rupture, we also detected a neuroprotective effect of Nec-1 since we observed a reduction from 38.6 ± 2.2% to 26.5 ± 2.1% of the LDH release (Fig 3.1-E). Moreover, we observed that zVAD.fmk (20 μM), a broad caspase inhibitor, affected the apoptotic component of cell death reducing the number of apoptotic-like nuclei from 30.4 ± 6.2% to 21.1 ± 2.9% (Fig 3.1-C), but had no significant neuroprotective effect when we analyzed necrotic neuronal death (12.4 ± 1.0% of necrotic neurons in OGD compared to 16.5 ± 1.2% for OGD in the presence of zVAD.fmk) (Fig 3.1-A).

Recently, events of DR-independent necroptosis were described (Feoktistova et al., 2011; Tenev et al., 2011), so we tested whether a TNFα neutralizing antibody could influence cell death induced by OGD. We observed that by inhibiting TNFα signaling we were able to significantly protect neurons from OGD-induced death (LDH release was reduced from 24.2 ± 1.9% to 16.3 ± 1.4%) (Fig 3.1-F). This suggests that in neurons submitted to OGD, DR signaling may mediate the activation of necroptotic cell death.

Necroptosis in OGD-challenged Neurons is Promoted by Up-regulation of RIP3

In recent years many efforts have been made to clarify the mechanisms underlying necroptosis (Chan and Baehrecke, 2012). However, in neurons, the mechanism by which necroptosis is activated is not known. In order to investigate the

mechanism underlying the necroptotic component of neuronal death induced by OGD we analyzed the mRNA levels of two known regulators of necroptosis: RIP3, a known specific necroptosis player upon adequate stimuli (Cho et al., 2009; He et al., 2009; Zhang et al., 2009) (Fig 3.2-A) and caspase-8, which is a negative regulator of necroptosis (Oberst et al., 2011) (Fig 3.2-B). Interestingly, we found that RIP3 is significantly upregulated at 7 and 24h following OGD while caspase-8 is transiently downregulated 7h after OGD. These results suggest that a subset of neurons may downregulate apoptosis, while upregulating RIP3 expression, which may become afterwards more available for activation of necroptotic signaling.

To confirm that RIP3 mRNA levels translate to increased protein expression following OGD, we analyzed total RIP3 levels by western blotting. Indeed, at 24h after OGD we observed a significant increase in RIP3 protein expression, to about 1.5 fold of the control level (Fig 3.2-C, D), indicating that after OGD there is an increased availability of RIP3 that may facilitate its inclusion in the necrosome complex.

Since NMDARs are generally considered to be responsible for much of the neurodegeneration resultant from ischemic insults due to excitotoxic events mediated by these receptors (Deupree et al., 1996; Gee et al., 2006), we investigated the effect of NMDAR inhibition on the induction of RIP3 following OGD. We observed that the increase in RIP3 protein levels observed 24h after OGD was completely blocked by treatment with MK-801 (Fig 3.2-C, D), suggesting that activation of NMDARs during an OGD challenge contributes to the induction of necroptotic neuronal death. Indeed, MK-801 prevented hippocampal neuron death induced by OGD as assessed by the LDH release (data not shown).

To determine whether RIP3 upregulation following OGD affects the susceptibility of hippocampal neurons to the ischemic insult, we studied the effect of RIP3 overexpression as well as the effect of RIP3 KD on hippocampal neuron survival (Fig 3.3). To do so, we co-transfected rat hippocampal neurons with GFP plus RIP3 or the empty vector pBOB (Fig 3.3-A, B), and analyzed the PI positive nuclei in neurons submitted to OGD under fluorescence microscopy, to assess necrotic cell death in transfected neurons. We observed that overexpression of RIP3 significantly potentiated the damaging effect of the OGD challenge (Fig 3.3-A). Interestingly, overexpression of RIP3 did not induce toxicity *per se*, which implies that RIP3-mediated necrosis is only activated upon specific insults.

On the other hand, to confirm the contribution of RIP3 endogenous expression to cell death we performed KD experiments in mouse hippocampal neurons transfected

with GFP and a previously described shRNA targeting mRIP3 (Zhang et al., 2011) or a control shRNA against LacZ (Fig 3.3-C, E). We observed that RIP3 KD significantly protected hippocampal neurons against the OGD insult (Fig 3.3-C). The specificity of this effect was addressed through a rescue experiment, by co-transfecting neurons with the shRNAs plus a hRIP3 construct, which is not recognized by the mouse shRNA used in this work (Fig 3.3-D, E). The expression of hRIP3 rescues the toxic effect of the OGD challenge (Fig 3.3-D), thereby confirming the specificity of the protective effect of endogenous RIP3 KD. These results highlight that the up-regulation of RIP3 mRNA and protein following OGD has a detrimental effect, contributing to neuronal death by necrosis.

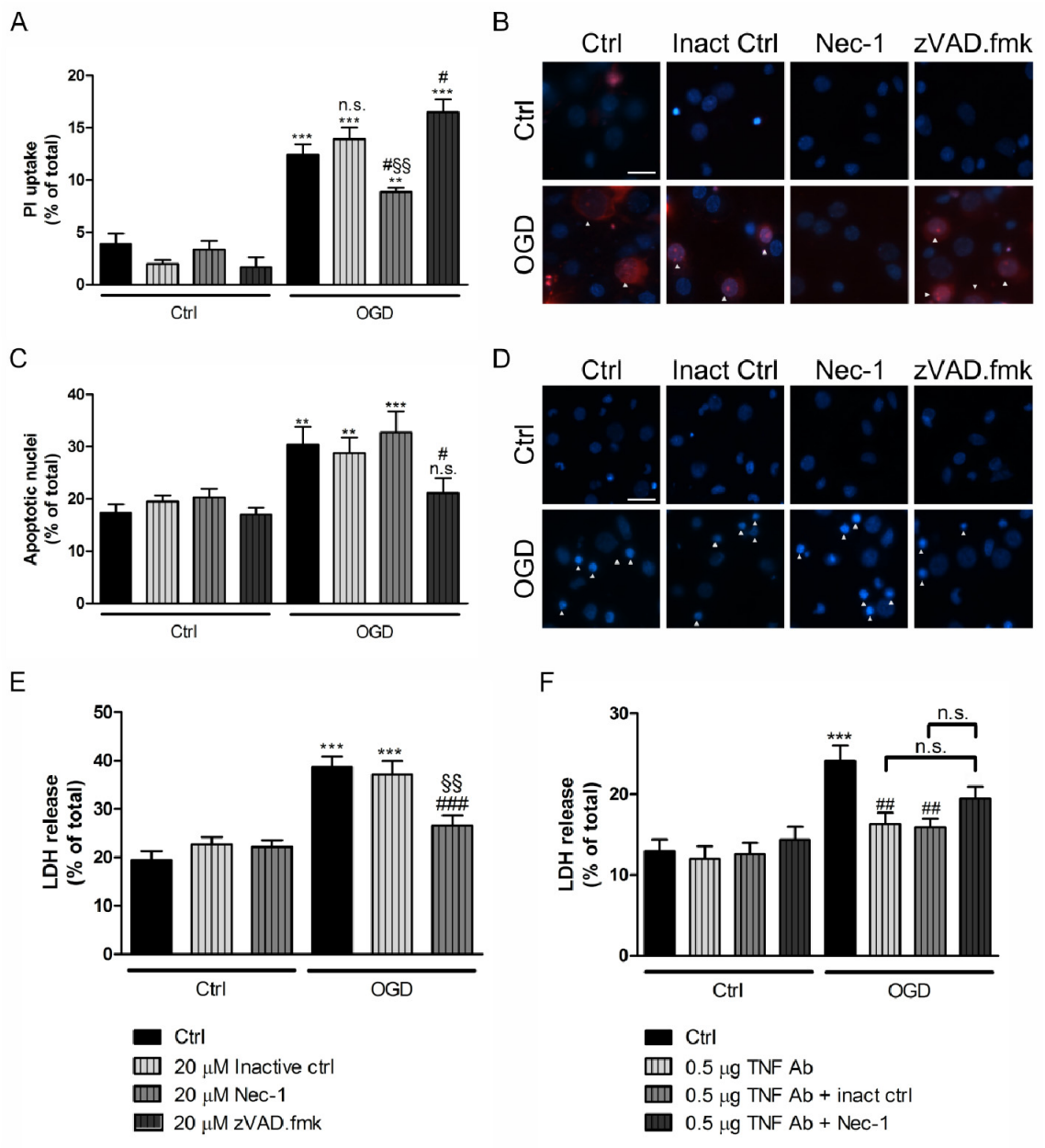


Figure 3.1. The OGD challenge induces necroptotic neuronal death. Mature cultures of hippocampal neurons (15 DIV) were challenged with OGD for 2h. Twenty four hours after the insult, cell viability was assessed. The necroptosis inhibitor Nec-1, its inactive control and the broad caspase inhibitor zVAD.fmk were added 1h prior to stimulation and kept during the stimulation and post-stimulation periods. **(A)** Analysis of PI staining of OGD challenged hippocampal neurons. The results were expressed as the percentage of necrotic neurons (PI positive cells without pyknosis - white arrowheads) relatively to the total number of cells. Bars represent the mean ± SEM of 9 independent experiments (n=4 for zVAD.fmk conditions). **(B)** Representative images of data shown in A. Scale bar – 20 μm. **(C)** Analysis of the nuclear morphology of hippocampal neurons, with Hoechst 33342 staining. The results were expressed as the percentage of apoptotic-like cells relatively to the total number of cells. Bars represent the mean ± SEM of 9 independent experiments (n=7 for zVAD.fmk conditions). **(D)** Representative images of data shown in C. Scale bar – 20 μm. **(E)** The LDH activity was assayed as indicated in the manufacturer's protocol. Bars represent the mean ± SEM of 12 independent experiments (n=6 for zVAD.fmk conditions). **(F)** A TNFα neutralizing antibody partially prevents the cell death induced by OGD, suggesting the activation of DRs in this system. The TNF antibody was added 1h prior to OGD (0.5 μg/ml) and kept for the remainder of the experiment. The LDH activity was assayed as indicated in the manufacturer's protocol. Bars represent the mean ± SEM of 8 independent experiments (n=6 for Nec-1 conditions). *Significantly different from control (One way ANOVA followed by Bonferroni's multiple comparison test: *$p<0.05$, **$p<0.01$, ***$p<0.001$, relatively to Control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, relatively to OGD and $^{\S}p<0.05$, $^{\S\S}p<0.01$, relatively to inactive control).

RIP1 Contributes to Necroptotic Neuronal Death Following OGD

A plethora of studies has established a determinant role for both RIP1 and RIP3 in necroptotic signaling. In fact, they are thought to interact through their RIP homotypic interaction motif (RHIM) domains, forming a complex which is responsible for activation of cell death by necroptosis upon specific stimulation (Sun et al., 2002; Cho et al., 2009; He et al., 2009; Zhang et al., 2009). To address whether RIP1 could also be induced in our model of ischemic injury, we analyzed the RIP1 protein content 24h after OGD, a time point for which we observed increased RIP3 expression (Fig 3.2). Indeed, 24h after OGD, the amount of RIP1 is significantly increased (Fig 3.4-A, B). As observed for RIP3, the induction of RIP1 protein levels was correlated with the activation of neuronal death, since overexpression of RIP1 significantly contributed to necrotic cell death induced by the OGD insult, compared to the empty vector (Fig 3.4-

C, D). Thus, both RIP1 and RIP3 contribute to necrotic cell death in hippocampal neurons challenged with OGD.

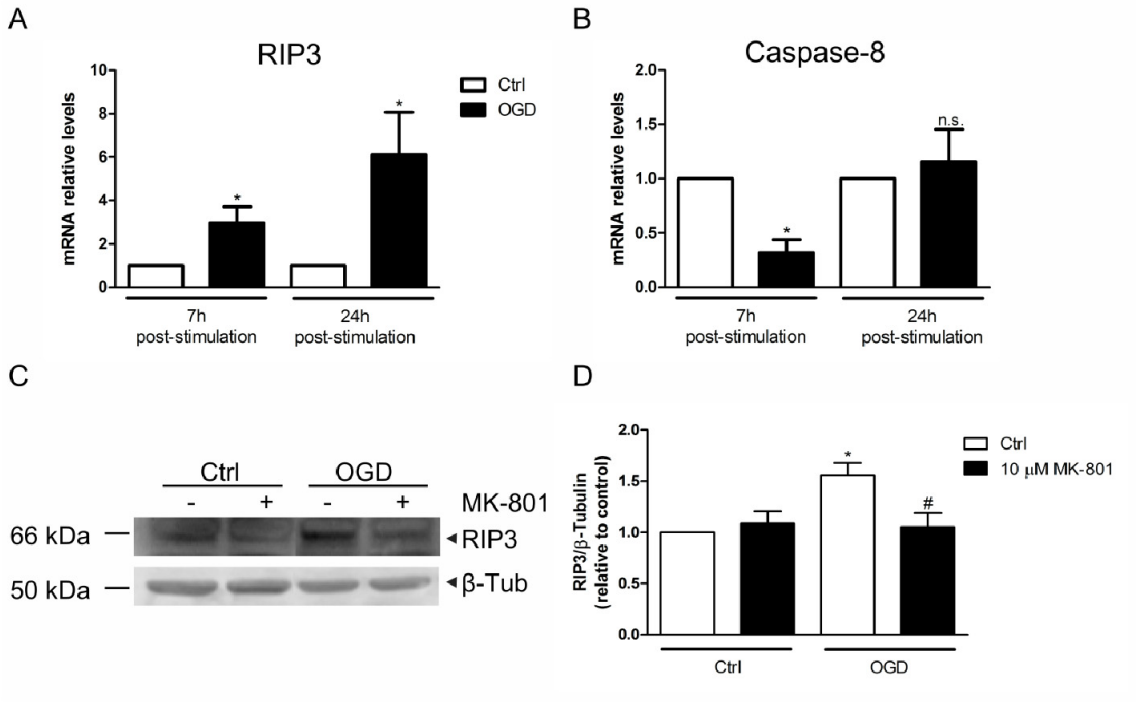


Figure 3.2. OGD induces the expression of the necroptosis-related RIP3 protein. Mature cultures of hippocampal neurons (15 DIV) were challenged with OGD for 2h. **(A, B)** Seven and 24 hours after OGD, total RNA was extracted with TriZol. Real-time PCR analysis was performed using cDNA prepared from 1 µg of total RNA and the primers for RIP3 (n=4 independent experiments) (A) and Caspase-8 (7h: n=5; 24h: n=4 independent experiments) (B). The fold change in mRNA levels was normalized to GAPDH and β-Actin. *Significantly different from control (*$p<0.05$, Student's t-test on log-transformed data). **(C)** Total protein extracts were collected 24h after 2h of OGD and western blot analysis was performed for total RIP3. The NMDAR antagonist, MK-801 (10 µM) was added to the cells 15 min prior to OGD and kept during the stimulation and post-stimulation periods. MK-801 abolishes the OGD-induced RIP3 up-regulation. **(D)** Bars represent the mean ± SEM of 4 independent experiments. *Significantly different from control [**$p<0.01$ (compared to control), $^{\#}p<0.05$ (compared to OGD), One-way ANOVA followed by Bonferroni's multiple comparison test].

RIP3 is Upregulated Following Global Cerebral Ischemia in vivo

Although several brain ischemia paradigms have been suggested to have a component of necroptotic neuronal death, based on the neuroprotective effect of Nec-1 (Degterev et al., 2005; Xu et al., 2010; Northington et al., 2011), to date, changes in the

protein levels of the necrosome components have not been reported. To investigate whether the changes in RIP1 and RIP3 observed in our *in vitro* model of ischemic injury reflect modifications that might occur *in vivo*, we submitted adult rats to transient global cerebral ischemia (Fig 3.5). We observed that RIP3 is indeed upregulated 48h after global ischemia, specifically in the CA1 region of the hippocampus, while it remained constant in the CA3 and DG areas, and in the cerebral cortex (Fig 3.5-A, B). RIP1, on the other hand, displayed a trend to a slight increase in the CA1, and DG areas of the hippocampus, although without statistical significance (Fig 3.5-A, C). We also observed that the upregulation of RIP3 in the CA1 area correlated with increased formation of α-Spectrin break-down products (SBDPs) of 145 and 150 kDa, demonstrating the activation of calpains, which are proteases involved in cell death, namely in neuronal death induced by ischemic insults (Siman et al., 1989; Vosler et al., 2011). These results suggest that global ischemia induces the upregulation of necrosome components in CA1 hippocampal neurons, which is probably related to the activation of necroptotic neuronal death in this paradigm.

Discussion

Recently, necroptosis has been extensively studied as a novel type of programmed cell death and it has been implicated in a wide range of pathologies, including cerebral ischemia. Using an *in vitro* model of global cerebral ischemia, OGD, we showed that this challenge induces necroptotic neuronal death. Interestingly, we found that OGD upregulated both RIP1 and RIP3 protein levels, which we found to occur also *in vivo*, following a transient global cerebral ischemic insult, at least for the RIP3 protein. Furthermore, we were able to correlate the upregulation of endogenous RIP3 with an increase in necrosis of hippocampal neurons following OGD.

Apoptosis is widely accepted as one of the major forms of programmed cell death, requiring the sequential activation of biochemical reactions, namely those leading to the formation of the apoptosome and the activation of caspases, as a result of the release of proapoptotic proteins from the mitochondria (reviewed in Taylor et al., 2008; Andersen and Kornbluth, 2013). On the contrary, necrosis is generally thought of as an “accidental” type of cell death that occurs when the cell is submitted to an overwhelming stress that afterwards leads to the activation of an inflammatory response (Kanduc et al., 2002). This view of the necrotic cell death as a systematic

unregulated event has been challenged by studies showing that apoptotic inhibitors (such as broad caspase inhibitors like zVAD.fmk) do not always protect but instead they may sensitize the cells to a mechanism of programmed necrosis (Vercammen et al., 1998; Holler et al., 2000). Additionally, it was found that in caspase-$8^{-/-}$ mice, which die around E10.5, cells are sensitized to necrotic cell death, in response to DR activity (Oberst et al., 2011), uncovering a role of caspase-8 as a negative regulator of programmed necrosis or necroptosis. The role of DR signaling in activation of programmed cell death mechanisms, including necroptosis, has been extensively studied (reviewed in Dickens et al., 2012).

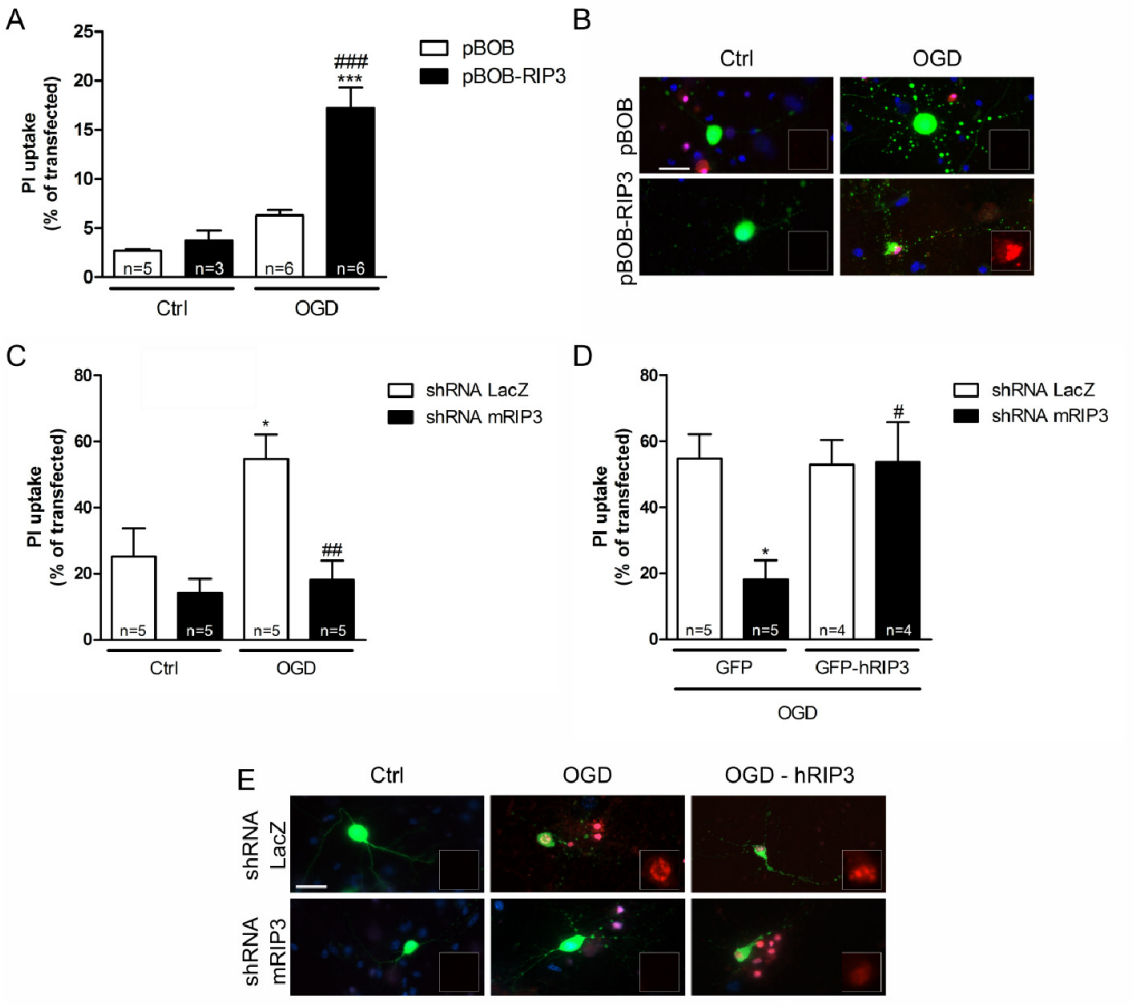


Figure 3.3. The levels of the RIP3 protein influence the extent of OGD-induced neuronal death. (A-B) Primary cultures of rat hippocampal neurons were co-transfected at DIV 7 with the indicated constructs (3 µg) plus a GFP plasmid (1.5 µg), using the calcium phosphate protocol. At DIV 15 cells were challenged with OGD for 2h. Twenty four hours after stimulation cells were stained with the nuclear dyes Hoechst 33342 and PI and the number of PI positive transfected cells was counted. The results were expressed as the percentage of PI positive cells relatively to the total number of

transfected cells. **(A)** Overexpression of RIP3 significantly enhances OGD toxicity in hippocampal neurons. **(B)** Representative images of data shown in A. Scale bar – 20 μm; Insert – 10 μm. **(C-E)** Primary cultures of mouse hippocampal neurons were co-transfected at DIV 7 with the indicated constructs (1.5 μg) plus a GFP plasmid (0.75 μg) or hRIP3 (1.5 μg), using the calcium phosphate protocol. At DIV 15 cells were challenged with OGD for 2h. Twenty four hours after stimulation cells were stained as in (A) and the number of PI positive transfected cells was counted. The results were expressed as the percentage of PI positive cells relatively to the total number of transfected cells. **(C)** Knock-down of RIP3 significantly protects neurons against OGD-induced death (n=5). **(D)** In OGD-challenged neurons, the neuroprotective effect of RIP3 KD is reversed by expression of hRIP3, which is not recognized by the mRIP3 shRNA. **(E)** Representative images of data shown in C and D. Scale bar – 20 μm; Insert – 10 μm. Bars represent the mean ± SEM of the indicated number of independent experiments. *Significantly different from control [One way ANOVA followed by Bonferronni's multiple comparison test: *$p<0.05$, ***$p<0.001$ relative to control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ relative to OGD condition (A) or OGD-LacZ (C and D)].

In an effort to elucidate the involvement of necroptosis in pathologies with broad relevance, Nec-1 was identified as a specific inhibitor of necroptosis (Degterev et al., 2005). More recently, Nec-1 was found to target the RIP1 kinase activity (Degterev et al., 2008). This inhibitor has been shown to be protective against a variety of cellular stresses, both in neurons (Li et al., 2008; Wang et al., 2012) as well as in non-neuronal cells (Upton et al., 2010; Narayan et al., 2012; Simenc and Lipnik-Stangelj, 2012). Moreover, this inhibitor of necroptosis was shown to be protective against focal ischemic injury *in vivo* (Degterev et al., 2005; Northington et al., 2011; Chavez-Valdez et al., 2012) and OGD *in vitro* (Xu et al., 2010; Meloni et al., 2011). Accordingly, we also observed a component of neuronal death that was inhibited by Nec-1 in our *in vitro* model of global cerebral ischemia. Additionally, we demonstrated that Nec-1 protected neurons from OGD-induced necrosis, without affecting the component of apoptotic-like cell death. Furthermore, the broad caspase inhibitor zVAD.fmk was only effective in reducing apoptotic-like neuronal death, whilst having no protective effect against necrotic neuronal death evaluated with PI staining. In this case, zVAD.fmk actually exacerbated the neuronal death induced by OGD, which is in accordance with previous reports showing that inhibition of caspases favors necroptotic cell death (Vercammen et al., 1998). We also observed that TNFα signaling contributes to hippocampal neuronal death induced by OGD, probably contributing to the activation of the extrinsic pathway of apoptosis as well as necroptosis. We were not able to detect a significant

effect of Nec-1 when neurons were co-treated with the TNFα neutralizing antibody and the necroptosis inhibitor since the TNFα neutralizing antibody provided a complete neuroprotection in our experimental conditions, suggesting that the induction of necroptosis in hippocampal neurons subjected to OGD might be mediated by TNFR1 activation.

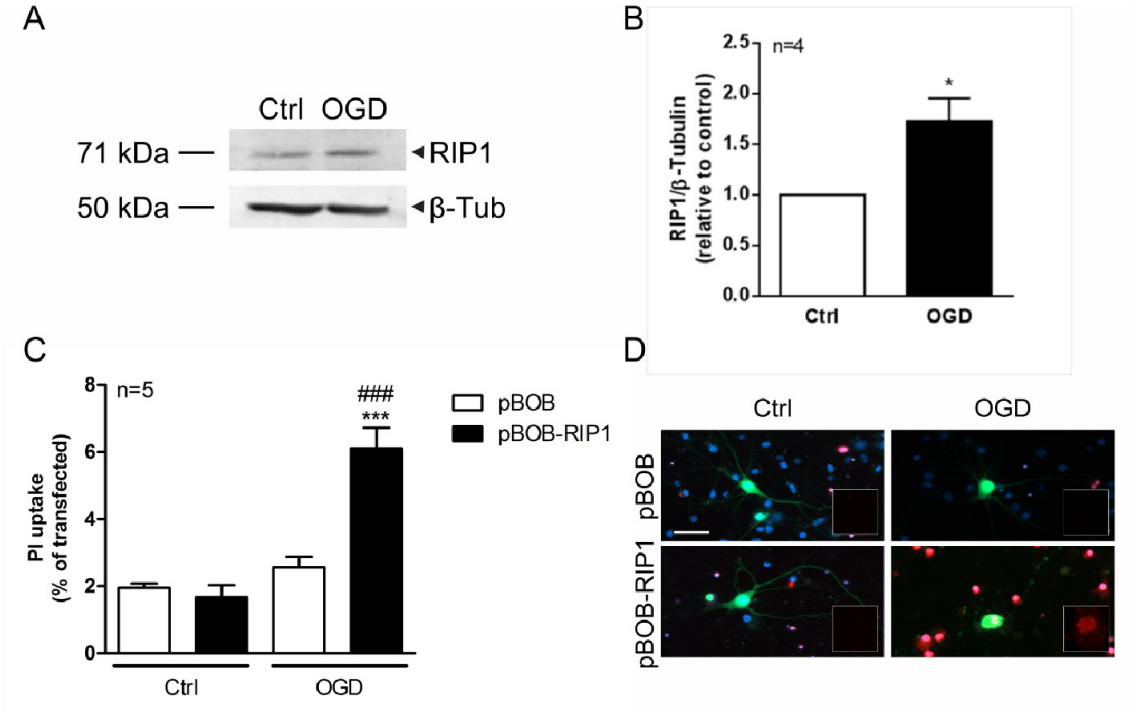


Figure 3.4. RIP1 protein levels are induced following OGD. Mature cultures of hippocampal neurons (15 DIV) were challenged with OGD for 2h. **(A-B)** Total protein extracts were collected 24h after 2h of OGD and western blot analysis was performed for total RIP1 (A). RIP1 is significantly up-regulated following OGD (B). Bars represent the mean ± SEM of the indicated number of independent experiments. *Significantly different from control (*p<0.05, ***p<0.001, Student's t-test). **(C-D)** Primary cultures of rat hippocampal neurons were co-transfected at DIV 7 with the indicated constructs (3 μg) plus a GFP plasmid (1.5 μg), using the calcium phosphate protocol. At DIV 15 cells were challenged with OGD for 2h. Twenty four hours after stimulation cells were stained with the nuclear dyes Hoechst 33342 and PI and the number of PI positive transfected cells was counted. The results were expressed as the percentage of PI positive cells relatively to the total number of transfected cells. **(C)** Overexpression of RIP1 significantly enhances OGD toxicity in hippocampal neurons. **(D)** Representative images of data shown in C. Scale bar – 20 μm; insert – 10 μm. *Significantly different from control [***p<0.001 (relative to control); $^{###}$p<0.001 (relative to OGD-pBOB condition), One way ANOVA followed by Bonferronni's multiple comparison test.]

The identification of a necroptotic cell death program in a subpopulation of hippocampal neurons submitted to OGD prompted us to investigate the mechanisms that might regulate necroptosis upon an ischemic stimulus, since the events underlying neuronal necroptotic death have not been elucidated so far. In non-neuronal systems, the interaction between the related proteins RIP1 and RIP3 as well as their kinase activity has been shown to be crucial for necroptotic cell death (Cho et al., 2009; He et al., 2009; Zhang et al., 2009; Li et al., 2012), while caspase-8 may act as a negative regulator possibly by promoting the cleavage of both RIP1 and RIP3 (Feng et al., 2007; Cho et al., 2009; Oberst et al., 2011). Our results indicate that in a subset of neurons OGD induces a transient down-regulation of caspase-8 mRNA while increasing RIP3 mRNA levels. The transcriptional changes induced by OGD are paralleled by an increase in the endogenous RIP3 level. The endogenous RIP1 protein level is increased as well, suggesting these events may underlie the formation of the necrosome upon a toxic stimulus. Furthermore, we identified NMDAR signaling as an important regulator of RIP3 levels, which may explain the occurrence of necroptosis following NMDAR activation (Li et al., 2008). Importantly, we also observed increased levels of RIP3 in the CA1 region of the hippocampus of rats submitted to transient global cerebral ischemia *in vivo*. This region of the hippocampus is considered to be selectively vulnerable to this type of insult (Kirino, 1982; Zukin et al., 2004) and our results suggest that necroptosis contributes to the neurodegeneration that ensues in that area upon global cerebral ischemia. Moreover, our results demonstrate the relevance of the induction of endogenous RIP3 protein to the toxicity elicited by global cerebral ischemia, both *in vitro* and *in vivo*. This is, to our knowledge, the first report describing increased RIP3 expression following cerebral ischemic paradigms.

To more accurately establish the role of the RIP3 and RIP1 proteins in the induction of necroptosis in hippocampal neurons submitted to OGD, we overexpressed both proteins in this system. Overexpression of either RIP1 or RIP3 resulted in the exacerbation of OGD-induced neuronal death. Importantly, we observed that the increase in the extent of cell death in transfected neurons occurred specifically upon OGD challenge since control neurons overexpressing RIP3 or RIP1 did not present significant changes in cell viability. This implies that regulated necrosis requires specific stimuli to be activated and that overexpression of RIP3 or RIP1 by themselves does not induce cell death. This is in accordance with a previous report, that demonstrated that overexpression of RIP3 and RIP1 specifically induces cell death only upon necroptotic stimulation (Zhang et al., 2009).

The determinant role of RIP3 in necroptosis has been demonstrated by complete abrogation of necrotic cell death in RIP3-depleted cells (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). Similarly, we observed that RIP3 KD in hippocampal neurons abolished necrotic death induced by OGD (Fig 3). Importantly, we observed that by reintroducing RIP3 in KD neurons we rescued the necrotic component of cell death elicited by OGD. These results highlight the crucial impact of RIP3 expression levels on OGD-induced necrosis and support evidence showing an increase in necrotic cell death due to the expression of RIP3 in cells normally resistant to necroptotic stimuli (Zhang et al., 2009).

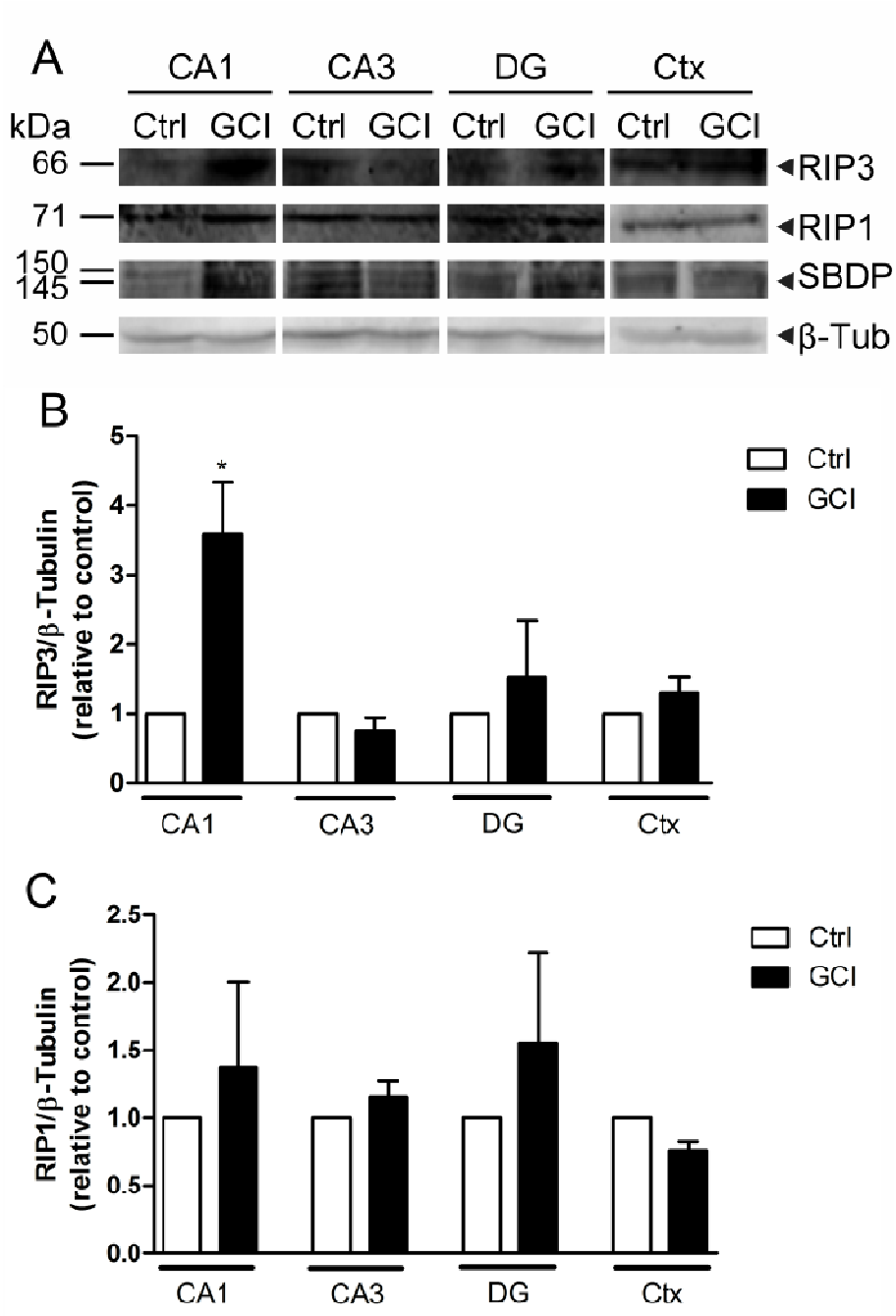


Figure 3.5. Global cerebral ischemia induces expression of the RIP3 protein. Adult rats were challenged with in vivo transient global cerebral ischemia (GCI) for 15 min. Forty-eight hours after GCI, the rats were sacrificed and the hippocampus and cortex Ctx were dissected. The hippocampus was further dissected to separate the CA1, CA3 and DG regions. **(A)** Total protein extracts were collected and western blot analysis was performed for RIP3, RIP1, SBDP (to demonstrate activation of cell death pathways), and β-tubulin. **(B)** GCI induced an upregulation of RIP3 in the CA1 region of the hippocampus. Bars represent the mean ± SEM of 3 independent experiments (n=4 for the Ctx). *Significantly different from control (*$p<0.05$, Student's t-test on log-transformed data). **(C)** The RIP1 protein was not changed following GCI. Bars represent the mean ± SEM of 3 independent experiments (n=2 for the DG).

Overall, our results support the view that ischemic insults induce a plethora of cell death mechanisms, including apoptosis and programmed necrosis (Meloni et al., 2011). The understanding of the diverse death mechanisms that occur in ischemia may prove beneficial to the identification of novel molecular targets relevant to the development of new treatments for this pathology. Additionally, our results demonstrate the induction of both RIP1 and RIP3 proteins following OGD, as well as of RIP3 mRNA. The relevance of this upregulation of RIP3 is further demonstrated by an increase in RIP3 protein levels in the CA1 region of the hippocampus, following an *in vivo* global ischemic insult. Further studies concerning the mechanisms of necroptosis in neurons, namely the identification of putative RIP1/3 substrates, possibly neuron-specific, may provide important insights to clarify the mechanisms of cell death underlying global ischemic insults.

Chapter IV

Activation of MAPK Signaling Following *in vitro* Ischemic Insults

Abstract

Global cerebral ischemia, that results from a lack of blood supply to the entire brain, is characterized by selective neurodegeneration of specific subsets of neurons, namely in the hippocampus. This type of insult can be mimicked *in vitro* by combined oxygen and glucose deprivation (OGD), in primary neuronal cultures or slices. Mitogen-activated protein kinases (MAPKs) have been widely studied for their role in the response to cellular stress and were found to be involved in a variety of cell death paradigms, namely in neurons. In this work we addressed whether OGD induces the activation of the MAPKs c-Jun N-terminal kinase (JNK) and p38. We observed that indeed, we were able to detect increased activation of both kinases, at distinct time points after OGD. We observed increased p38 phosphorylation at 2h of recovery and higher levels of the classical JNK substrate c-Jun at 6h post-stimulation. Additionally, we also observed altered mRNA levels of the JNK and p38 pathways substrates *Madd*, *Atf3* and *Gadd45a* and upstream kinases *Ask2* and *Tpl2*. To confirm their role in neuronal death induced by OGD, we used the p38 inhibitor, SB203580 and the JNK cascade inhibitors, SP600125 and CEP11004 and observed that all these compounds conferred neuroprotection against OGD. Interestingly, we observed that the JNK signaling inhibitors were only neuroprotective when applied 4h after the end of the OGD challenge but not when present throughout the stimulation and post-stimulation periods. This effect suggests that JNK may have a dual role in the regulation of neuronal fate under OGD conditions. Overall our results highlight the role of MAPKs in the context of cerebral ischemia. Inhibition of these kinases may provide important benefits for the treatment of this type of insult.

Introduction

Cerebral ischemia is one of the most prevalent neuropathologies worldwide (Flynn et al., 2008). Transient global ischemia results from a lack of blood supply to the entire brain, commonly due to a cardiac arrest. This blood deprivation induces the selective death of specific subsets of neurons in the brain, of which the CA1 region of the hippocampus is one of the most prominently affected (Zukin et al., 2004). This type of insult induces delayed neuronal death that occurs 24-72h following the insult, for which there are to date, no effective treatments. Currently, research in this area aims at reducing the amount of neurodegeneration that ensues as a consequence of ischemic

insults. Thus, there is great interest on the understanding of the toxic intracellular signaling pathways that are activated in these conditions, in order to identify new molecular targets useful for therapy.

The mitogen-activated protein kinases (MAPKs) have a variety of functions in cells, including neurons, namely a crucial impact on the life and death balance. These signaling pathways have a typical three-tiered cascade, comprising a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK. Each sequential kinase is activated by phosphorylation and they exert their role in the cell by phosphorylating a variety of substrates, depending on the cellular context. There are three groups of mammalian MAPKs - extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) – and they have been all correlated to the response to cellular stress (Qi and Elion, 2005). However, while ERK1/2 is commonly associated with cell survival (Liu et al., 1998; Liu et al., 2013), the MAPKs p38 and JNK are thought to contribute to cell death, namely in neurons (Ham et al., 1995; Yang et al., 1997; Borsello et al., 2003; Cao et al., 2004; Semenova et al., 2007; Lee et al., 2013). In particular, both kinases have been correlated with neurodegeneration induced by ischemic insults, both *in vitro* (Behrens et al., 1999; Hirt et al., 2004) and *in vivo* (Ferrer et al., 1997; Sugino et al., 2000; Borsello et al., 2003; Kuan et al., 2003; Hirt et al., 2004; Okuno et al., 2004). One of the preferential JNK substrates is the transcription factor c-Jun (Gupta et al., 1996). This transcription factor homodimerizes or heterodimerizes with other members of the Jun, activating transcription factor (ATF) and Fos families, assembling activator protein-1 (AP-1) transcription factor complexes, which mediate the transcription-dependent toxic effects of JNK activation (Ham et al., 1995; Davis, 2000; Hess et al., 2004; Silva et al., 2005; Santos et al., 2006; Burke, 2007).

In this work, we aimed to study the activation of p38 and JNK in hippocampal neurons submitted to oxygen and glucose deprivation (OGD), an *in vitro* model of cerebral ischemia. We observed that both pathways are activated following OGD, but at different time points. Interestingly, our results suggest that JNK may be activated in a biphasic manner, since we observed that we were only able to confer neuroprotection with JNK inhibitors when the compounds were added at 4h of reoxygenation, but no neuroprotection was observed when the compounds were present during the stimulation and post-stimulation periods. This suggests that prior to a toxic activation that we detected at 6h of recovery, JNK may be involved in a prosurvival mechanism. This hypothesis supports previous observations concerning JNK activation mediated by tumor necrosis factor receptor 1 (TNFR1) signaling (Ventura et al., 2006) or induced by

cerebral ischemia injury (Hirt et al., 2004) and excitotoxic signaling (Vieira et al., 2010). Moreover, we show that this biphasic activation mediates differential effects on the neuronal fate following OGD.

Materials & Methods

Materials

The antibodies for phospho-c-Jun (Ser63) (#9261) and total p38 (#9212) were purchased from Cell Signaling Technology (Boston, MA). The antibodies for c-Jun/AP-1 (sc-45-G) and p-p38 (Thr 180/Tyr 182-R) (sc-17852-R) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

The JNK pathway inhibitor CEP11004 was a generous gift from from Cephalon, Inc. (West Chester, PA) and SP600125 was purchased from Sigma-Aldrich (St. Louis, MO). The p38 inhibitor SB203580 was purchased from Calbiochem (Merck-Millipore, Billerica, MA).

Cell Culture

Primary cultures of hippocampal neurons were prepared from embryonic day 18 (E18) rats, as previously described (Santos and Duarte, 2008). Briefly, hippocampi were dissected and dissociated with trypsin (1.5 mg/ml) in Hank's Balanced Salt Solution (HBSS). Neurons were cultured in multiwell (MW) plates with or without glass coverslips, coated with poly-D-lysine (PDL), at a density of 85,000 cells/cm^2, in Neurobasal® medium (Gibco®, Life Technologies™, Paisley, UK) supplemented with 2% NeuroCult™ SM1 Neuronal Supplement (StemCell™ Technologies, Grenoble, France), 0.5 mM glutamine, 0.125 µg/ml gentamicin and 25 µM glutamate. Neuronal cultures were maintained at 37ºC in a humidified incubator with 5% CO_2 up to 15 days *in vitro* (DIV). At DIV 7, the cultures were fed with fresh supplemented neurobasal medium without glutamate.

Oxygen-Glucose Deprivation (OGD) Challenge

At DIV 15, hippocampal neurons were challenged with an *in vitro* ischemic stimulus. For that purpose, neurons were placed in a glucose-free deoxygenated buffer medium (OGD medium – 10 mM HEPES, 116 mM NaCl, 5.4 mM KCl, 0.8 mM NaH_2PO_4, 25 mM sodium bicarbonate, 25 mM sucrose, 1.8 mM $CaCl_2$, 0.04% phenol red, pH 7.3), inside an anaerobic chamber with 5% CO_2 (OGD chamber, Thermo Forma 1029, Thermo Fisher Scientific, Waltham, MA), at 37ºC for 2h. Control neurons were placed in a similar buffer, containing 25 mM glucose, instead of sucrose (sham medium), and kept for 2h in an oxygenated incubator, at 37ºC. After incubation, the neurons were placed in their conditioned medium and returned to the normoxic incubator for a period of recovery that lasted up to 24h.

When indicated, JNK or p38 inhibitors were added to the cell medium. SB203580 was pre-incubated for 1h and then kept during the stimulation and post-stimulation periods. For inhibition of the JNK signaling pathway, CEP11004 and SP600125 were added 1h prior to stimulation and then kept during the stimulation and post-stimulation periods or, alternatively, the inhibitors were added 4h after the OGD insult and kept for the remainder of the post-stimulation period.

RNA extraction

Total RNA from DIV 15 hippocampal neurons was extracted with TRIzol® Reagent (Invitrogen, Madrid, Spain), following the manufacturers' specifications. The total amount of RNA was quantified spectrophotometrically (Thermo Nanodrop 2000, Thermo Fisher Scientific, Wilmington, DE) and the RNA quality and integrity evaluated using the Experion automated gel electrophoresis system (BioRad, Hercules, CA).

qPCR

For cDNA synthesis, 1 µg of total RNA was used with the iScript™ cDNA Synthesis Kit (BioRad), according to the manufacturers' instructions. For quantitative real-time PCR (qRT-PCR) 20 µl reactions were prepared with 2 µl of 1:10 diluted cDNA, 10 µl of 2x iQ™ SYBR® Green Supermix (BioRad) and specific primers at 250 nM. Primer sequences are indicated in Table II.

Table II. Primers used for qPCR analysis.

Gene	Forward Primer	Reverse Primer	Annealing Temperature
Actb	5'-CGTCACCTACTCTAACCG-3'	5'-CTTGTGCTATCTGCTCATC-3'	54ºC
Ask2	5'-ACAACTATACAAGCTCAATGC-3'	5'-GGGTCTGAAATGCTGGTA-3'	54.5ºC
Atf3	5'-GATGGAGTGAGAACATTCAT-3'	5'-AGGACAGAACAGCATAGT-3'	55ºC
Gadd45a	5'-TTACTCAAGCAGTCACTC-3'	5'-CTCCTGTAGTTGAACTCA-3'	56ºC
Gapdh	5'- AACCTGCCAAGTATGATG-3'	5'-GGAGTTGCTGTTGAAGTC-3'	55ºC
Madd	5'-AAGTCTCTCTGCGATGAA-3'	5'-GATCACTAGATAGTCAAGTAACC-3'	55ºC
Taok3	5'-GAAAGACAAGAGCGAGAG-3'	5'-AAATCTAATGTCACCAAATTCC-3'	54ºC

The fluorescent signal was measured after each elongation step of the PCR reaction, in the iQ5 Multicolor Real-Time PCR Detection System (BioRad), and was used to determine the threshold cycle (C_t), as previously described (Manadas et al., 2009). Melting curves were performed in order to detect non-specific amplification products, a non-template control was included in all assays, and for each set of primers a standard curve was performed to assess primer efficiency. Reactions were run in duplicate. The level of expression of each gene was analyzed at 7 and 24h of recovery following OGD and normalized to the level of expression of the gene in the corresponding control conditions. All C_t values were normalized to two internal control genes, ActB and Gapdh, using the GenEx software (MultiD Analyses).

Nuclear morphology

Neuronal death was assessed by analysis of the nuclear morphology 24h following the OGD insult. For that purpose, neurons were stained with the nuclear dye Hoechst 33342 (2 µg/ml) for 15 min at RT and then mounted in fluorescent mounting medium. Neurons were analyzed under fluorescence microscopy for chromatin condensation and nuclear pyknosis, a feature of apoptotic-like cells. The results were expressed as the percentage of apoptotic-like cell death (apoptotic-like nuclei/total number of cells). At least 200 cells were counted in each coverslip.

Lactate Dehydrogenase (LDH) release assay

At 24h of recovery after the OGD challenge, the LDH release to the extracellular medium was evaluated by a colorimetric assay using the CytoTox96 Non-Radioactive assay kit (Promega, Madison, WI), according to the manufacturer's instructions. The

percentage of LDH release was determined as the ratio between LDH activity in the extracellular medium and total LDH activity obtained after complete cell lysis with Triton X-100. All experiments were carried out in duplicate or triplicate, for each independent experiment, as previously described (Vieira et al., 2010).

Protein Extracts, SDS-PAGE and Western Blotting

Twenty four hours after OGD, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysis buffer [50 mM HEPES, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 2 mM Na3VO4, 50 mM NaF, 1% Triton X-100, 10% glycerol, pH 7.4 supplemented with 1 mM phenylmethanesulphonylfluoride (PMSF) and 1 µg/ml of CLAP (cocktail containing chymostatin, leupeptin, antipain and pepstatin)] was added. Cell extracts were then sonicated and centrifuged for 20 min at 12000 x g, at 4ºC. Supernatants were recovered and the protein quantified by the BCA method. Samples were denatured at 95ºC, for 10 min and 80 µg of each protein extract were separated by SDS-PAGE, using 10% polyacrilamide gels, and then transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with Tris-buffered saline-Tween20 (TBS-T) (20 mM Tris, 137 mM NaCl, pH 7.6, 0.1 % Tween20) with 5% non-fat milk, for 1h at room temperature, and then incubated with the primary antibody in TBS-T 5% milk, for 1h at room temperature. Alternatively, the incubation with the primary antibody was performed overnight at 4°C. After extensive washing, membranes were incubated with the secondary antibody conjugated with alkaline phosphatase for 1h at room temperature. After additional washes, the membranes were developed using the enhanced chemifluorescence (ECF) substrate, and scanned on the Storm 860 Gel and Blot Imaging System (Amersham Biosciences, Buckinghamshire, UK). The density of the bands was analyzed with ImageQuant 5.0 software. For subsequent reprobing, the membranes were stripped of antibody with NaOH 0.2 M, blocked again and incubated with the appropriate antibodies.

Statistical Analysis

Results are presented as means ± S.E.M. of the indicated number of experiments. Statistical significance was assessed by Student's *t*-test or by one-way variance analysis (ANOVA) followed by the Dunnett's test or the Bonferroni's test. These statistical analyses were performed using the software package GraphPad Prism 5.

Results

OGD Induces Up-regulation of the JNK signaling Pathway

The MAPK JNK has been shown to have a prominent role in the activation of cell death upon several stress insults (Eilers et al., 1998; Tournier et al., 2000; Cao et al., 2004; Hunot et al., 2004; Kukekov et al., 2006; Ventura et al., 2006; Wang et al., 2013), including excitotoxicity (Yang et al., 1997; Borsello et al., 2003; Centeno et al., 2007; Vieira et al., 2010) and cerebral ischemia (Ferrer et al., 1997; Kuan et al., 2003; Hirt et al., 2004; Okuno et al., 2004; Gao et al., 2005). Thus, we addressed whether our *in vitro* model of global ischemia, OGD, could induce the activation of the JNK signaling pathway. To address this question, we looked at changes on c-Jun phosphorylation, a classical JNK substrate, which is considered to be a measure of altered JNK activity. We tested different times of OGD and recovery and observed that c-Jun phosphorylation appeared to be up-regulated following 6h of reoxygenation after OGD (Fig 4.1-A). Since this was the only time point for which we observed an apparent increase in c-Jun phosphorylation we quantified the blots for 6h of recovery and confirmed that c-Jun was significantly up-regulated after 6h of recovery following 2h of OGD to 1.29 ± 0.09 times of the control (Fig 4.1-B). The phosphorylation of c-Jun is known to up-regulate its transcriptional activity, as c-Jun complexes with other members of the Jun and Fos families to form the AP-1 transcription factor (Bohmann et al., 1987; Santos et al., 2006), and to regulate transcription of genes related to cell death (Herdegen and Waetzig, 2001; Hess et al., 2004). To confirm that the increase in p-c-Jun observed following OGD correlates with an increased transcriptional activity of this protein, we analyzed changes in the mRNA level of some of the genes it may regulate, namely the *Atf3*, which is known to be induced by c-Jun/AP-1 (Liang et al., 1996; Mei et al., 2008) (Fig 4.1-C), the growth arrest and DNA damage 45a (*Gadd45a*, Fig 4.1-D), also known to be up-regulated by JNK activity (Yin et al., 2004), and the MAP-kinase activating death domain (*Madd*, Fig 4.1-E), which is down-regulated upon activation of JNK (Del Villar and Miller, 2004). As observed by qRT-PCR analysis, both the *Atf3* and *Gadd45a* genes had a higher expression 7h and 24h after OGD, respectively, while *Madd* expression was decreased at both time points. These results suggest that c-Jun might be indeed activating gene transcription following OGD.

Since JNK activity may be initiated by a variety of MAPKKKs, depending on the specific stimulus (Davis, 2000), we also analyzed the changes induced by OGD in the expression of a few MAPKKKs, by qRT-PCR. We observed that both apoptosis signal-regulating kinase 2 (*Ask2*) (Fig 4.2-A) and tumor progression locus 2 (*Tpl2*) (Fig 4.2-B)

had a higher expression, especially at 24h after OGD. On the contrary, we did not observe changes in the mRNA levels of TAO kinase 3 (*Taok3*) (Fig 4.2-C). These results suggest that ASK2 and TPL2 may be involved in hippocampal neuronal death induced by OGD.

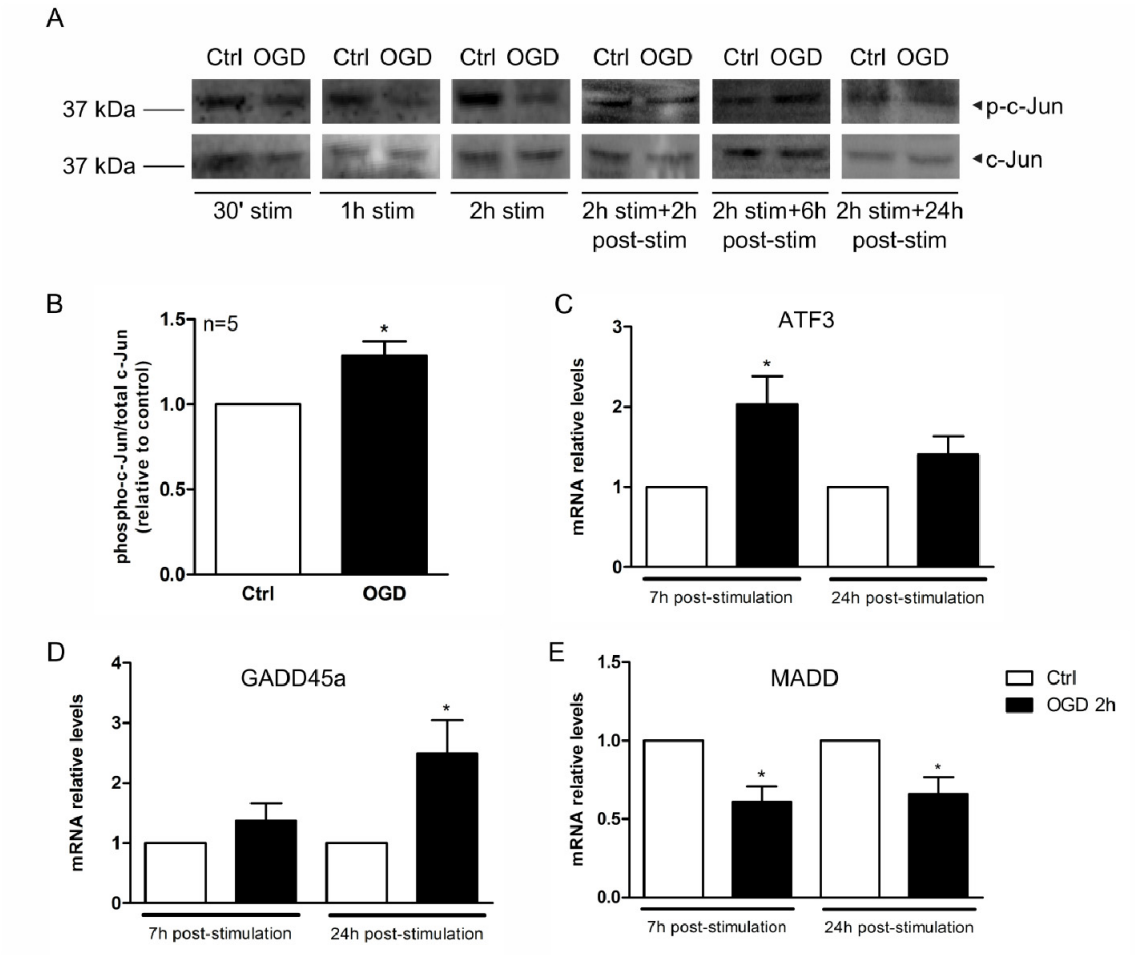


Figure 4.1. OGD induces changes in JNK pathway targets. (A) Mature cultures of hippocampal neurons (15 DIV) were challenged with OGD for different periods. At the end of the insult, protein extracts were immediately prepared or, alternatively, The cultures were transferred to the conditioned medium and returned to the air/CO_2 incubator for a period of reoxygenation that lasted up to 24h and total protein extracts were prepared. Eighty µg of total protein were used for immunoblot analysis with an anti-phospho-c-Jun antibody. The membranes were reprobed with an anti-total c-Jun antibody. The figure is representative of 2 (30 min OGD and 2h OGD + 24h recovery), 1 (1h OGD, 2h OGD and 2h OGD + 2h of recovery) or 5 (2h OGD + 6h recovery) assays performed with extracts from independent experiments. **(B)** Quantification of the ratio between phospho-c-Jun and total levels of c-Jun at 6h of recovery, normalized to the control. Bars represent the mean ± SEM of the indicated number of experiments (*p<0,05, Student's *t* test). **(C-E)** Seven and 24 hours after OGD, total RNA was extracted

with TriZol. Real-time PCR analysis was performed using cDNA prepared from 1 µg of total RNA and the primers for ATF3 **(C)**, GADD45a **(D)** and MADD **(E)**. Fold change in mRNA levels was normalized to GAPDH and β-Actin. Bars represent the mean ± SEM of 5 independent experiments. *Significantly different from control (*p<0.05, Student's *t*-test on log-transformed data).

JNK Inhibition Protects Against Neuronal Death

To confirm that the increase of JNK activity in our system is indeed correlated with neuronal death, we used two JNK cascade inhibitors – CEP11004 [a Mixed-lineage kinase (MLK) inhibitor] and SP600125 (a specific JNK inhibitor). Surprisingly, we observed that if we inhibited JNK signaling throughout the entire experiment (including both the OGD insult as well as the 24h of recovery), we could not detect any neuroprotection against the OGD challenge. However, if we added the compounds to the cells 4h after the end of OGD, we were able to significantly reverse the majority of cell death elicited by this challenge [from about 39.7 ± 4.7% to 18.2 ± 1.9% of cell death for CEP1104 and from about 38.6 ± 5.1% to 21.6 ± 2.5% of cell death for SP600125 (Fig 4.3)]. These results suggest that OGD may elicit two distinct phases of JNK activity, with different contributions to hippocampal neurons cell fate. This is in line with our previous work (Vieira et al., 2010), where we reported a biphasic JNK activation induced by excitotoxicity, a deleterious phenomenon also present in cerebral ischemia. We propose that the initial activation of JNK (prior to 4h of reoxygenation) may have a prosurvival effect, while the second phase of JNK activation results in increased c-Jun phosphorylation within 6h of recovery and contributes to neuronal death. Additionally, the neuroprotective effect afforded by CEP11004 suggests that MLK3 may be one of the MAPKKKs to induce JNK activation in this context.~

OGD Elicits Activation of the p38 MAPK

Another MAPK, p38, has been extensively studied for its role in induction of neuronal death following ischemia (Behrens et al., 1999; Sugino et al., 2000; Li et al., 2013). Thus, we addressed the question of whether this pathway is also activated in hippocampal neurons following OGD. We tested several times of OGD and recovery and observed a significant increase in p-p38 levels following OGD, to about 1.4 times of the control (Fig 4.4-A, B), at 2h of recovery following 2h of OGD. We did not detect increased c-Jun phosphorylation at this time point (Fig 4.1-A). We were also able to

correlate p38 activation with neuronal death since we observed that SB203580, a specific p38 inhibitor, afforded significant neuroprotection against the OGD challenge (Fig 4.4-C, D). The inhibitor SB203580 was able to reduce the cell death induced by OGD from about 31.6 ± 2.2% to 21.0 ± 2.7% on the LDH assay and from about 41.2 ± 3.7% to 28.7 ± 0.6% on the nuclear morphology analysis. Interestingly, p38 inhibition is protective if performed previously to OGD and through the recovery period, suggesting p38 activation by ischemic stimulus has mainly a deleterious role while JNK might have an initial prosurvival action and a later prodeath activity. Thus, the roles of these two MAPKs are not redundant, with p38 influencing only hippocampal neuronal death, whilst JNK may have dual roles in neuronal fate.

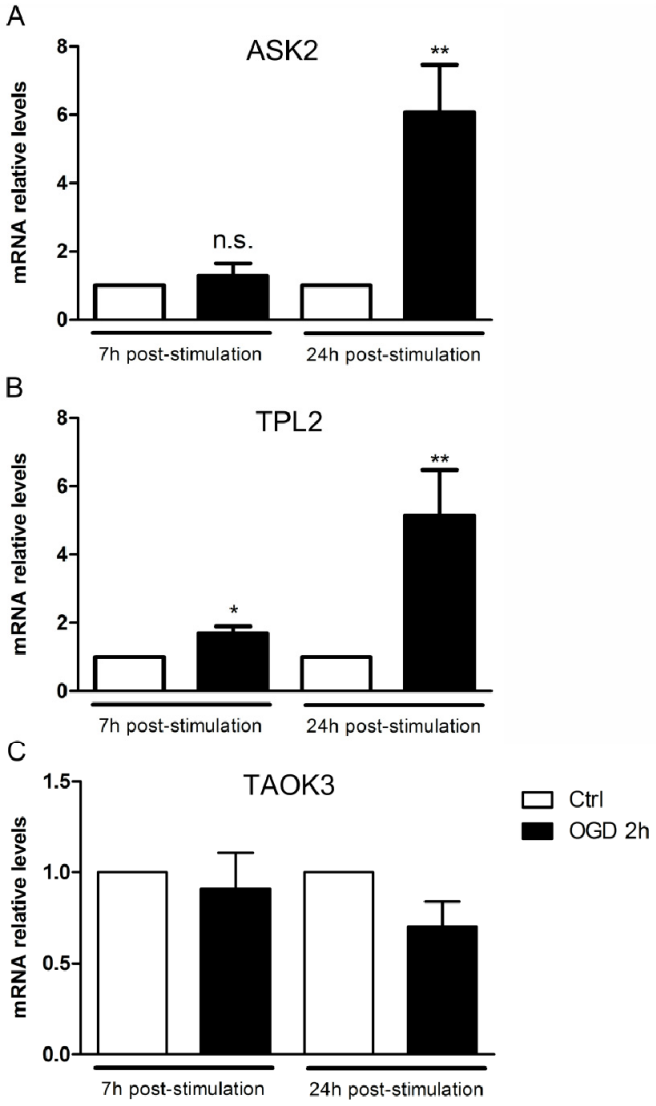


Figure 4.2. OGD up-regulates MAPKKKs of the JNK signaling pathway. Mature cultures of hippocampal neurons (15 DIV) were challenged with OGD for 2h. The cultures were then transferred to the conditioned medium and returned to the air/CO_2 incubator.

Seven and 24 hours after OGD, total RNA was extracted with TriZol. Real-time PCR analysis was performed using cDNA prepared from 1 µg of total RNA and the primers for ASK2 **(A)**, TPL2 **(B)** and TAOK3 **(C)**. Fold change in mRNA levels was normalized to GAPDH and β-Actin. Bars represent the mean ± SEM of 5 independent experiments. *Significantly different from control (*p<0.05, **p<0.001, Student's *t* test on log-transformed data).

Discussion

The MAP kinases are hallmark signaling pathways in stress response, namely in cerebral ischemia insults. We tested whether these kinases, specifically p38 and JNK, were activated by an *in vitro* model of cerebral global ischemia, OGD. We observed that OGD induced an increase in the phosphorylation of p38 and of the classical JNK substrate, c-Jun. We also detected changes in the mRNA levels of c-Jun target genes and other JNK substrates as well as in a few MAPKKKs. Notably, we also report significant neuroprotection afforded by a p38 inhibitor. Interestingly, we were also able to protect neurons from OGD-induce damage but only when we applied the inhibitors of JNK signaling 4h after the insult. This result suggests that JNK may have a dual effect, contributing both to neuron survival as well as demise. Overall our results highlight the importance of these signaling pathways in the neuronal response to *in vitro* ischemia.

The MAPK signaling, mostly through p38 and JNK MAPKs, has been extensively studied in recent years for its role in cell fate upon a plethora of stress insults, including cerebral ischemia (Ferrer et al., 1997; Behrens et al., 1999; Sugino et al., 2000; Harper and LoGrasso, 2001; Borsello et al., 2003; Kuan et al., 2003; Hirt et al., 2004; Okuno et al., 2004). These kinases have a typical three-tiered signaling cascade regulated by phosphorylation. Upon induction of a specific stimulus, a MAPKKK is activated, usually by autophosphorylation, giving rise to the phosphorylation of a more specific MAPKK. This protein then phosphorylates a specific MAPK that may target a wide variety of substrates (Weston and Davis, 2007). JNK has been correlated with a plethora of apoptotic cell death paradigms, both in neurons (Ham et al., 1995; Yang et al., 1997; Coffey et al., 2002; Borsello et al., 2003; Cao et al., 2004; Centeno et al., 2007) and in non-neuronal cells (Tournier et al., 2000; Bonny et al., 2001; Lei and Davis, 2003). Although all JNK isoforms can induce cell death

(Bjorkblom et al., 2008), JNK3, a brain specific isoform has been demonstrated to be essential for the neurodegeneration that ensues following excitotoxic and ischemic insults (Yang et al., 1997; Kuan et al., 2003). On the other hand, the p38 MAPK also mediates relevant signaling pathways of neuronal death (Kawasaki et al., 1997; Behrens et al., 1999; Sugino et al., 2000; Cao et al., 2004; Segura Torres et al., 2006; Semenova et al., 2007; Lee et al., 2013). Nevertheless, JNK and p38 are argued to mediate distinct apoptotic paradigms (Cao et al., 2004). In this work we observed that both kinases are activated following OGD, in hippocampal neurons. Notably, the peak of activation of each kinase was distinct. However, we report a concomitant role of both kinases upon *in vitro* ischemia, as previously reported for *in vivo* insults (Ozawa et al., 1999). This may imply a redundant mechanism, to ensure neuronal death upon extensive cellular damage.

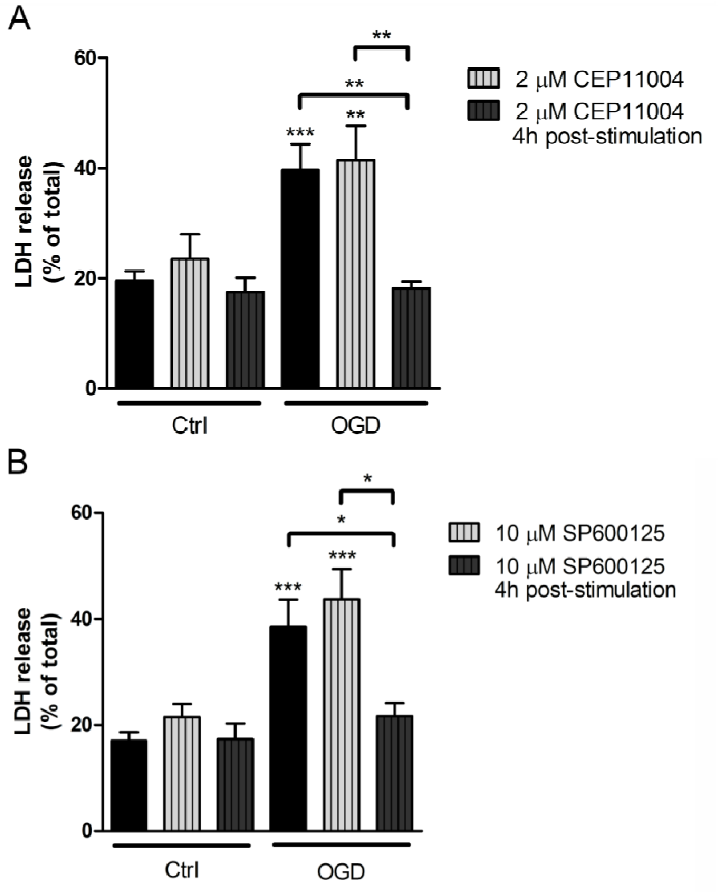


Figure 4.3. JNK has dual effects on neuronal fate following the OGD challenge. Mature cultures of hippocampal neurons (15 DIV) were challenged with OGD for 2h. Twenty four hours after OGD, cell viability was assessed. The JNK pathway inhibitors CEP11004 **(A)** and SP600125 **(B)** were added 1h prior to stimulation and kept during the stimulation and post-stimulation periods or, alternatively, were added 4h after the OGD insult and kept for the remainder of the reoxygenation period. The LDH activity was

assayed as indicated in the manufacturer's protocol. Bars represent the mean ± SEM of 10 independent experiments (n=4; n=7). *Significantly different from control (*p<0.05, **p<0.01, ***p<0.001, One way ANOVA followed by Bonferroni's multiple comparison test).

The MAPK signaling, mostly through p38 and JNK MAPKs, has been extensively studied in recent years for its role in cell fate upon a plethora of stress insults, including cerebral ischemia (Ferrer et al., 1997; Behrens et al., 1999; Sugino et al., 2000; Harper and LoGrasso, 2001; Borsello et al., 2003; Kuan et al., 2003; Hirt et al., 2004; Okuno et al., 2004). These kinases have a typical three-tiered signaling cascade regulated by phosphorylation. Upon induction of a specific stimulus, a MAPKKK is activated, usually by autophosphorylation, giving rise to the phosphorylation of a more specific MAPKK. This protein then phosphorylates a specific MAPK that may target a wide variety of substrates (Weston and Davis, 2007). JNK has been correlated with a plethora of apoptotic cell death paradigms, both in neurons (Ham et al., 1995; Yang et al., 1997; Coffey et al., 2002; Borsello et al., 2003; Cao et al., 2004; Centeno et al., 2007) and in non-neuronal cells (Tournier et al., 2000; Bonny et al., 2001; Lei and Davis, 2003). Although all JNK isoforms can induce cell death (Bjorkblom et al., 2008), JNK3, a brain specific isoform has been demonstrated to be essential for the neurodegeneration that ensues following excitotoxic and ischemic insults (Yang et al., 1997; Kuan et al., 2003). On the other hand, the p38 MAPK also mediates relevant signaling pathways of neuronal death (Kawasaki et al., 1997; Behrens et al., 1999; Sugino et al., 2000; Cao et al., 2004; Segura Torres et al., 2006; Semenova et al., 2007; Lee et al., 2013). Nevertheless, JNK and p38 are argued to mediate distinct apoptotic paradigms (Cao et al., 2004). In this work we observed that both kinases are activated following OGD, in hippocampal neurons. Notably, the peak of activation of each kinase was distinct. However, we report a concomitant role of both kinases upon *in vitro* ischemia, as previously reported for *in vivo* insults (Ozawa et al., 1999). This may imply a redundant mechanism, to ensure neuronal death upon extensive cellular damage.

Interestingly, we observed that, whilst inhibition of p38 during the OGD challenge and the whole recovery period is neuroprotective, JNK signaling seems to be more complex since neuroprotection was achieved only when the JNK inhibitor was added 4h after the stimulus. Indeed, JNK inhibition continuously during OGD and the recovery period abrogated any possible neuroprotective effect. A similar effect was

reported by Hirt et al. (2004) in organotypic hippocampal slices submitted to OGD. These authors observed that applying D-JNKI-1 at 3h or 9h after OGD had no protective effect, but administration 6h after the insult was neuroprotective (Hirt et al., 2004). Thus, our results suggest the occurrence of a dual role of JNK, with two distinct phases of activity. Accordingly, JNK has been suggested to have a role both in neuronal survival as well as neuronal death (Waetzig et al., 2006; Weston and Davis, 2007), depending on its cellular localization (Coffey et al., 2000; Bjorkblom et al., 2008). Accordingly, in our OGD model, the cytoplasmic pool of JNK may be activated in an initial phase during the insult or early after the end of OGD, contributing to neuronal survival while the activation of the nuclear JNK pool, contributing to neuronal death, may only occur at later time points. In fact, there are evidences that some cell death paradigms induce a biphasic JNK activation (Vieira et al., 2010) with distinct impact on cellular fate (Ventura et al., 2006; Kenchappa et al., 2010), as suggested in our study.

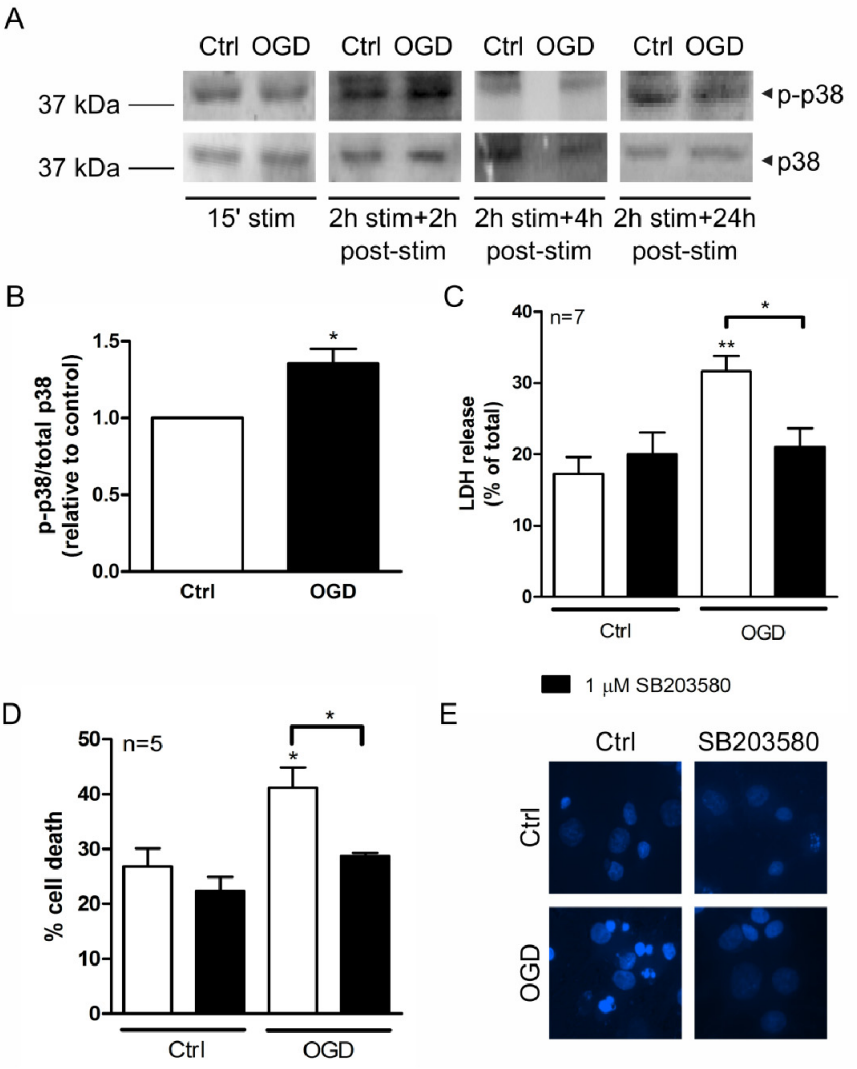


Figure 4.4. OGD induces p38 activation. (A) Mature cultures of hippocampal neurons (15 DIV) were challenged with OGD for 15 min or 2h. The cultures challenged with 2h of OGD were then transferred to the conditioned medium and returned to the air/CO_2 incubator for a period of time that lasted up to 24h. At the indicated times, total cell extracts were prepared. Eighty µg of total protein were used for immunoblot analysis with an anti-phospho-p38 antibody. The membranes were reprobed with an anti-pan-p38 antibody. The figure is representative of 2 (15min, 4h and 24h) or 5 (2h of recovery) assays performed with extracts from independent experiments. **(B)** Quantification of the ratio between phospho-p38 and total levels of p38, normalized to the control. Bars represent the mean ± SEM of the indicated number of experiments ($p<0,05$). **(C)** The LDH activity was assayed at 24h post-stimulation as indicated in the manufacturer's protocol. Bars represent the mean ± SEM of 7 independent experiments. **(D)** Analysis of the nuclear morphology of hippocampal neurons. The results were expressed as the percentage of apoptotic cells relatively to the total number of cells. Bars represent the mean ± SEM of 7 independent experiments. *Significantly different from control (*$p<0.05$, **$p<0.01$, One way ANOVA followed by Bonferroni's multiple comparison test).

We also investigated changes in the expression of putative MAPKKKs that might be responsible for activation of the JNK and p38 signaling pathways, to better clarify the specific cascade that contributes to neuronal death. ASK2 is a MAPKKK that forms heteromeric complexes with the related protein ASK1, leading to stress-induced JNK activation (Wang et al., 1998; Takeda et al., 2007). To date there are no evidences for a role of ASK2 in ischemia-induced neuronal death, but our results suggest a significant expression of the *Ask2* gene. TPL2 is a common MAPKKK to p38, JNK and ERK (Salmeron et al., 1996; Chiariello et al., 2000), but seems to be more correlated with physiological functions of these kinases (Hagemann et al., 1999). In fact, TPL2 seems to have a prosurvival function, by targeting Bim for degradation upon phosphorylation by ERK (Banerjee et al., 2008). Although we observed the upregulation of the mRNA of both ASK2 and TPL2, it remains to be clarified whether this translates into increased protein level and kinase activation. On the other hand, the neuroprotective effect of CEP11004 suggests that MLKs may be responsible for the activation of JNK in our ischemic paradigm, which correlates with evidence in the literature for a role of this MAPKKK in neurons (Xu et al., 2001; Tian et al., 2003; Zhu et al., 2012).

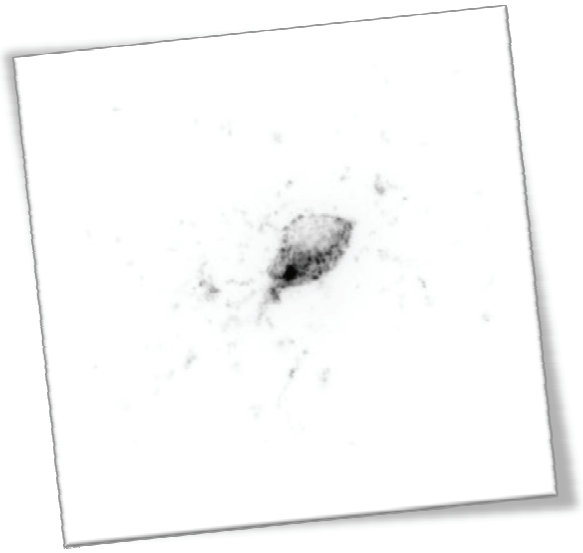
The classical effector of the transcription-dependent cell death mediated by JNK, the transcription factor c-Jun, activates the transcription of deleterious genes,

such as caspase-3 (Song et al., 2011) and Dp5 (Ma et al., 2007; Towers et al., 2009). Additionally to an increase in c-Jun phosphorylation, we observed an increase of *Atf3* gene expression, which has been shown to have ATF and AP-1 binding sites and to be regulated by the transcription factor c-Jun (Liang et al., 1996), which keeps in view with the idea of an increase of c-Jun activity upon the OGD challenge. Moreover, JNK has also been shown to up-regulate the mRNA and protein expression of GADD45α (Yin et al., 2004), which was also observed in our system for the mRNA, thus suggesting JNK activation following OGD. In fact, *gadd45a* mRNA has been shown to be altered following excitotoxic stimulation and cerebral ischemia (Hou et al., 1997; Zhu et al., 1997; Laabich et al., 2001). We also observed a decrease in MADD mRNA following the OGD challenge, which agrees with the decrease observed for the MADD protein as a consequence of NMDA excitotoxicity (Centeno et al., 2007) or in Alzheimer's disease (AD) (Del Villar and Miller, 2004). Both in neurons insulted with Aβ1-42 or in AD transgenic mice, MADD mRNA and protein levels are decreased and this effect is correlated with neuronal death. MADD interacts, in physiological conditions, with TNFR1 and JNK3 and the decrease in its protein levels is speculated to be a mechanism that allows TRADD interaction with TNFR1 and subsequent neuronal apoptosis (Del Villar and Miller, 2004). This protein is a known JNK substrate that translocates to the nucleus upon ischemic insults (Zhang et al., 1998).

Overall, our results demonstrate that both JNK and p38 are activated following the OGD insult. While p38 activation seems to contribute to neuronal death, the activation of JNK seems to have dual effects. This hypothesis is supported by the lack of neuroprotection observed when JNK inhibitors are present throughout the stimulation, implying that an initial activation of JNK may contribute to neuronal survival probably through the phosphorylation of endogenous substrates other than the nuclear pool of c-Jun. The activation of c-Jun within 6h of recovery with concurrent neuroprotection afforded by the JNK inhibitors when applied during the post-stimulation period suggests that after an initial period JNK activity is pro-survival, hence the duality of JNK function within the scope of cell fate upon ischemic stimulation.

Chapter V

General Conclusions and Future Directions



Conclusions

Cerebral ischemia is a major cause of disability and morbidity in the world (Flynn et al., 2008). Global cerebral ischemia, resultant from a lack of blood supply to the entire brain, induces selective neurodegeneration of the CA1 region of the hippocampus. Research efforts aim at the understanding of the molecular mechanisms underlying the neurodegeneration associated with cerebral ischemia. However, to date, effective treatments to global cerebral ischemia are lacking and the mechanisms underlying this pathology are still not completely understood. Thus, the goal of this work was the identification of the molecular mechanisms that induce neurodegeneration of hippocampal neurons following global ischemia using an *in vitro* model, OGD. For that purpose, we studied the neuronal demise induced by OGD by addressing different steps of the death process.

First, we studied the mechanism of excitotoxicity mediated by NMDARs, in particular the role of the GluN2B subunit in this process. We observed that OGD induces a component of excitotoxic neuronal death that is prevented by antagonism of NMDARs with the general inhibitor MK-801 and with ifenprodil, an antagonist of GluN2B-containing NMDARs. The determinant role of this subunit was further evidenced by the absence of toxicity in GluN2B$^{-/-}$ neurons that was rescued upon reintroduction of the subunit in these neurons. Although these receptors have long been recognized as important contributors to neuronal toxicity, the cause for the distinct effects of the GluN2A and GluN2B subunits remains elusive. While GluN2A is proposed by several reports to have a role in neuronal survival, GluN2B is most commonly correlated with neuronal death upon excitotoxic insults. One of the suggested regulators of the function of GluN2 subunits to neuronal response to excitotoxic challenges is the C-terminal domain, which is the most distinct region between the two subunits. To address this question we introduced a GluN2B subunit with the C-terminal domain swapped for that of GluN2A and observed that this subunit did not have the ability to rescue the toxicity induced by OGD. This effect corroborates a recent report that adopted a similar strategy to uncover the distinct roles of the GluN2 subunit in a context of excitotoxicity (Martel et al., 2012). In this work, we used a more physiological model, OGD that resembles more closely the effects of *in vivo* ischemia, thus demonstrating the crucial role of the C-terminus of the GluN2B subunit to ischemic damage. To further characterize the C-terminal domain of the GluN2B subunit, we treated WT neurons with a cell-permeable peptide that targets the interaction of the C-terminal domain of GluN2B with PSD95. This peptide significantly reduced the

neuronal death induced by OGD. This peptide has already been shown to have a neuroprotective effect against *in vivo* ischemia models (Aarts et al., 2002). Notably, we achieved a similar effect by expressing a GluN2B subunit with a mutation in the PDZ binding site in GluN2B$^{-/-}$ neurons, which is the domain responsible for this interaction. Interestingly, our findings demonstrate that other domains besides the PDZ binding site are important for GluN2B toxicity. We observed that subunits with mutations in the CaMKII-and the AP2-binding domains fail to evoke the toxicity of the WT GluN2B subunit. Ultimately, our results support the view that the GluN2B subunit has a determinant role in induction of neuronal death induced by ischemic insults. The selectivity of this subunit seems to reside on its C-terminal domain, which is highly divergent from that of the GluN2A subunit namely in respect to the established interactions with intracellular proteins.

Next, we studied a novel type of cell death, called necroptosis, in a context of OGD. Although some studies point to the occurrence of a component of necroptosis in the neurodegeneration induced by cerebral ischemia (Degterev et al., 2005; Xu et al., 2010; Northington et al., 2011), this evidence is mostly based on the neuroprotective effect of Nec-1, a necroptotic inhibitor. Thus, the specific molecular mechanism of necroptosis in neurons is yet to be clarified. We observed that Nec-1 was neuroprotective in our system, suggesting that OGD induces a component of necroptotic neuronal death. This mechanism relies on DR signaling and is activated in conditions where caspase-8, a negative regulator of necroptosis, is down-regulated or inhibited (Weinlich et al., 2011). When these conditions are gathered, RIP3 is recruited to a complex containing RIP1 and together they assemble the necrosome. We observed that, in hippocampal neurons submitted to OGD, the mRNA levels of caspase-8 are transiently decreased and concomitantly, RIP3 mRNA levels are induced. Furthermore, we observed that both necrosome proteins, RIP1 and RIP3 are upregulated 24h following OGD. This suggests that OGD may induce necroptosis by upregulating the proteins responsible for this cell death mechanism. We confirmed the toxic role of RIP3 induction in neurons by KD of the endogenous RIP3. This resulted in significant neuroprotection against the OGD challenge. Notably, we were able to rescue the damaging effect of OGD by expressing the human protein that is resistant to mouse shRNA against RIP3. Furthermore, we observed that overexpression of both RIP1 and RIP3 exacerbated the necrotic component of neuronal death induced by OGD. Our findings demonstrate the paramount role of RIP3 induction to the neuronal death initiated by OGD. These results gain relevance by the fact that RIP3 is also induced in the CA1 area of rats submitted to *in vivo* transient global ischemia. This

region of the hippocampus is considered to be selectively vulnerable to global ischemic insults and our results suggest that necroptosis might be one of the programmed cell death mechanisms that contribute to neurodegeneration in this area.

Finally, we studied the activation of death effector signaling pathways, in particular MAPKs. These proteins are widely associated with stress responses and are involved in several paradigms of cell death, including cerebral ischemia. In this work we addressed the activation of p38 and JNK and found that both pathways are activated. Accordingly, we observed changes in the mRNA levels of *Atf3*, *Madd* and *Gadd45a*, known substrates of JNK whose transcription may be mediated by c-Jun. Additionally, we observed changes in the mRNA levels of *Ask2* and *Tpl2*, which are MAPKKKs that may activate both JNK and p38 signaling pathways. Interestingly, whilst p38 activation appears to have a deleterious effect, JNK is suggested to have a dual effect in neuronal fate. We observed that inhibition of JNK was only neuroprotective if the compounds were added 4h after OGD, but not before. This implies that JNK may have an initial prosurvival role and a second phase of activation may be responsible for its role in neurodegeneration. Our previous work demonstrated a biphasic activation of JNK following excitotoxic stimulation of AMPARs (Vieira et al., 2010), and other studies support the notion of a dual role of JNK in response to certain stimuli (Hirt et al., 2004; Ventura et al., 2006). These results demonstrate that MAPKs are activated following *in vitro* ischemia, contributing to neuronal demise.

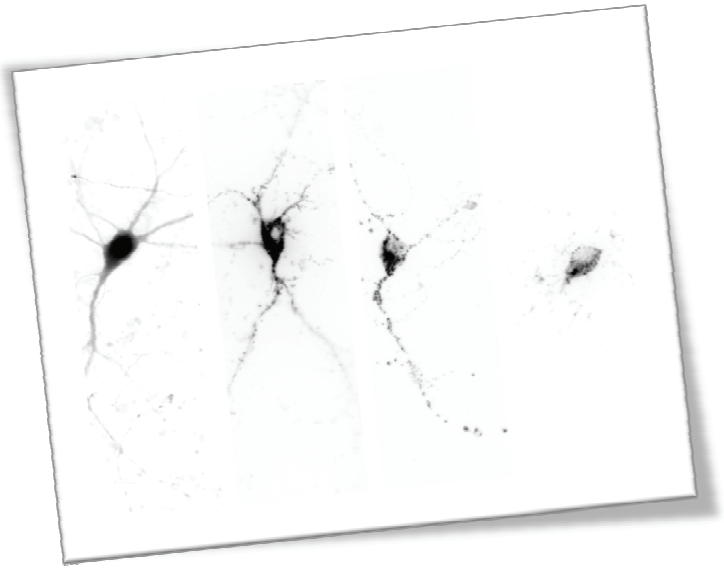
Overall, the work presented here demonstrates that ischemic insults activate a plethora of intracellular mechanisms that contribute to neuronal death. The multitude of effects that characterize this type of insult is evidenced by the fact that diverse sources of Ca^{2+} contribute to excitotoxic mechanisms (Arundine and Tymianski, 2003) and also that distinct mechanisms of cell death may occur, including apoptosis and necroptosis. Also, several effector pathways, including MAPKs and beyond, are activated culminating in neuronal demise. These facts highlight the importance of a broad understanding of the diverse mechanisms that are induced by ischemic insults and the value of the development of therapeutical strategies combining the inhibition of several deleterious intracellular mechanisms. Finally, this work demonstrates the avail of OGD as an *in vitro* ischemic model, since it can reproduce many of the molecular events underlying cerebral ischemia.

In the future, it will be important to validate some of the present findings *in vivo* and to further study some of the mechanisms implicated in neuronal death. For

example, regarding the NMDARs and the contribution of the GluN2B subunit, we describe three distinct domains in the C-terminus of this subunit that ablate neuronal death induced by OGD when mutated. Further work should be performed in order to better characterize the C-terminal domain of GluN2B and for a better understanding of the signaling pathways associated with these domains. Regarding the types of cell death that are activated following OGD, a combined action that prevents both apoptosis and necroptosis may prove beneficial and for that, a better understanding of the neuronal mechanisms of necroptosis is necessary, namely regarding the events downstream of the necrosome. This work focused on the understanding of the molecular mechanisms underlying *in vitro* ischemia, with the ultimate goal of preventing the neurodegeneration that ensues. Thus, in the future, these findings may contribute to the development of novel therapeutic strategies to cerebral ischemia.

Chapter VI

References



Aarts, M., Y. Liu, L. Liu, S. Besshoh, M. Arundine, J. W. Gurd, Y. T. Wang, M. W. Salter and M. Tymianski (2002). "Treatment of ischemic brain damage by perturbing NMDA receptor- PSD-95 protein interactions." Science **298**(5594): 846-50.

Al-Hallaq, R. A., T. P. Conrads, T. D. Veenstra and R. J. Wenthold (2007). "NMDA di-heteromeric receptor populations and associated proteins in rat hippocampus." J Neurosci **27**(31): 8334-43.

Al-Mubarak, B., F. X. Soriano and G. E. Hardingham (2009). "Synaptic NMDAR activity suppresses FOXO1 expression via a cis-acting FOXO binding site: FOXO1 is a FOXO target gene." Channels (Austin) **3**(4): 233-8.

Almeida, R. D., B. J. Manadas, C. V. Melo, J. R. Gomes, C. S. Mendes, M. M. Graos, R. F. Carvalho, A. P. Carvalho and C. B. Duarte (2005). "Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways." Cell Death Differ **12**(10): 1329-43.

Andersen, J. L. and S. Kornbluth (2013). "The tangled circuitry of metabolism and apoptosis." Mol Cell **49**(3): 399-410.

Araujo, I. M., B. P. Carreira, T. Pereira, P. F. Santos, D. Soulet, A. Inacio, B. A. Bahr, A. P. Carvalho, A. F. Ambrosio and C. M. Carvalho (2007). "Changes in calcium dynamics following the reversal of the sodium-calcium exchanger have a key role in AMPA receptor-mediated neurodegeneration via calpain activation in hippocampal neurons." Cell Death Differ **14**(9): 1635-46.

Arslan, S. C. and C. Scheidereit (2011). "The prevalence of TNFalpha-induced necrosis over apoptosis is determined by TAK1-RIP1 interplay." PLoS One **6**(10): e26069.

Arthur, P. G., G. P. Matich, W. W. Pang, D. Y. Yu and M. A. Bogoyevitch (2007). "Necrotic death of neurons following an excitotoxic insult is prevented by a peptide inhibitor of c-jun N-terminal kinase." J Neurochem **102**(1): 65-76.

Arundine, M. and M. Tymianski (2003). "Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity." Cell Calcium **34**(4-5): 325-37.

Arundine, M. and M. Tymianski (2004). "Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury." Cell Mol Life Sci **61**(6): 657-68.

Aurelian, L. (2005). "Cross talk of signaling and apoptotic cascades in the CNS: target for virus modulation." Front Biosci **10**: 2776-87.

Bading, H., D. D. Ginty and M. E. Greenberg (1993). "Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways." Science **260**(5105): 181-6.

Bading, H. and M. E. Greenberg (1991). "Stimulation of protein tyrosine phosphorylation by NMDA receptor activation." Science **253**(5022): 912-4.

Badiola, N., C. Malagelada, N. Llecha, J. Hidalgo, J. X. Comella, J. Sabria and J. Rodriguez-Alvarez (2009). "Activation of caspase-8 by tumour necrosis factor receptor 1 is necessary for caspase-3 activation and apoptosis in oxygen-glucose deprived cultured cortical cells." Neurobiol Dis **35**(3): 438-47.

Banerjee, A., R. Grumont, R. Gugasyan, C. White, A. Strasser and S. Gerondakis (2008). "NF-kappaB1 and c-Rel cooperate to promote the survival of TLR4-activated B cells by neutralizing Bim via distinct mechanisms." Blood **112**(13): 5063-73.

Bano, D., K. W. Young, C. J. Guerin, R. Lefeuvre, N. J. Rothwell, L. Naldini, R. Rizzuto, E. Carafoli and P. Nicotera (2005). "Cleavage of the plasma membrane Na+/Ca2+ exchanger in excitotoxicity." Cell **120**(2): 275-85.

Bantel, H., M. L. Schmitz, A. Raible, M. Gregor and K. Schulze-Osthoff (2002). "Critical role of NF-kappaB and stress-activated protein kinases in steroid unresponsiveness." FASEB J **16**(13): 1832-4.

Barbara, J. A., X. Van ostade and A. Lopez (1996). "Tumour necrosis factor-alpha (TNF-alpha): the good, the bad and potentially very effective." Immunol Cell Biol **74**(5): 434-43.

Barone, F. C., E. A. Irving, A. M. Ray, J. C. Lee, S. Kassis, S. Kumar, A. M. Badger, R. F. White, M. J. McVey, J. J. Legos, J. A. Erhardt, A. H. Nelson, E. H. Ohlstein, A. J. Hunter, K. Ward, B. R. Smith, J. L. Adams and A. A. Parsons (2001). "SB 239063, a second-generation p38 mitogen-activated protein kinase inhibitor, reduces brain injury and neurological deficits in cerebral focal ischemia." J Pharmacol Exp Ther **296**(2): 312-21.

Bayer, K. U., E. LeBel, G. L. McDonald, H. O'Leary, H. Schulman and P. De Koninck (2006). "Transition from reversible to persistent binding of CaMKII to postsynaptic sites and NR2B." J Neurosci **26**(4): 1164-74.

Behrens, M. M., U. Strasser, J. Y. Koh, B. J. Gwag and D. W. Choi (1999). "Prevention of neuronal apoptosis by phorbol ester-induced activation of protein kinase C: blockade of p38 mitogen-activated protein kinase." Neuroscience **94**(3): 917-27.

Benakis, C., C. Bonny and L. Hirt (2010). "JNK inhibition and inflammation after cerebral ischemia." Brain Behav Immun **24**(5): 800-11.

Bendotti, C., M. Tortarolo and T. Borsello (2006). "Targeting Stress Activated Protein Kinases, JNK and p38, as New Therapeutic Approach for Neurodegenerative Diseases." Central Nervous System Agents in Medicinal Chemistry **6**(2): 109-17.

Benveniste, E. N. and D. J. Benos (1995). "TNF-alpha- and IFN-gamma-mediated signal transduction pathways: effects on glial cell gene expression and function." FASEB J **9**(15): 1577-84.

Benveniste, M. and M. L. Mayer (1993). "Multiple effects of spermine on N-methyl-D-aspartic acid receptor responses of rat cultured hippocampal neurones." J Physiol **464**: 131-63.

Bernardi, A., R. L. Frozza, A. P. Horn, M. M. Campos, J. B. Calixto, C. Salbego, A. R. Pohlmann, S. S. Guterres and A. M. Battastini (2010). "Protective effects of indomethacin-loaded nanocapsules against oxygen-glucose deprivation in organotypic hippocampal slice cultures: involvement of neuroinflammation." Neurochem Int **57**(6): 629-36.

Bertrand, M. J., S. Milutinovic, K. M. Dickson, W. C. Ho, A. Boudreault, J. Durkin, J. W. Gillard, J. B. Jaquith, S. J. Morris and P. A. Barker (2008). "cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination." Mol Cell **30**(6): 689-700.

Besancon, E., S. Guo, J. Lok, M. Tymianski and E. H. Lo (2008). "Beyond NMDA and AMPA glutamate receptors: emerging mechanisms for ionic imbalance and cell death in stroke." Trends Pharmacol Sci **29**(5): 268-75.

Bianchi, K. and P. Meier (2009). "A tangled web of ubiquitin chains: breaking news in TNF-R1 signaling." Mol Cell **36**(5): 736-42.

Bidoret, C., A. Ayon, B. Barbour and M. Casado (2009). "Presynaptic NR2A-containing NMDA receptors implement a high-pass filter synaptic plasticity rule." Proc Natl Acad Sci U S A **106**(33): 14126-31.

Biton, S. and A. Ashkenazi (2011). "NEMO and RIP1 control cell fate in response to extensive DNA damage via TNF-alpha feedforward signaling." Cell **145**(1): 92-103.

Bjorkblom, B., J. C. Vainio, V. Hongisto, T. Herdegen, M. J. Courtney and E. T. Coffey (2008). "All JNKs can kill, but nuclear localization is critical for neuronal death." J Biol Chem **283**(28): 19704-13.

Blanpied, T. A., D. B. Scott and M. D. Ehlers (2002). "Dynamics and regulation of clathrin coats at specialized endocytic zones of dendrites and spines." Neuron **36**(3): 435-49.

Bogoyevitch, M. A. and B. Kobe (2006). "Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases." Microbiol Mol Biol Rev **70**(4): 1061-95.

Bohmann, D., T. J. Bos, A. Admon, T. Nishimura, P. K. Vogt and R. Tjian (1987). "Human proto-oncogene c-jun encodes a DNA binding protein with structural

and functional properties of transcription factor AP-1." Science **238**(4832): 1386-92.

Bonny, C., A. Oberson, S. Negri, C. Sauser and D. F. Schorderet (2001). "Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death." Diabetes **50**(1): 77-82.

Borsello, T., P. G. Clarke, L. Hirt, A. Vercelli, M. Repici, D. F. Schorderet, J. Bogousslavsky and C. Bonny (2003). "A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia." Nat Med **9**(9): 1180-6.

Boylan, J. M. and P. A. Gruppuso (1996). "A comparative study of the hepatic mitogen-activated protein kinase and Jun-NH2-terminal kinase pathways in the late-gestation fetal rat." Cell Growth Differ **7**(9): 1261-9.

Brancho, D., N. Tanaka, A. Jaeschke, J. J. Ventura, N. Kelkar, Y. Tanaka, M. Kyuuma, T. Takeshita, R. A. Flavell and R. J. Davis (2003). "Mechanism of p38 MAP kinase activation in vivo." Genes Dev **17**(16): 1969-78.

Brancho, D., J. J. Ventura, A. Jaeschke, B. Doran, R. A. Flavell and R. J. Davis (2005). "Role of MLK3 in the regulation of mitogen-activated protein kinase signaling cascades." Mol Cell Biol **25**(9): 3670-81.

Breder, C. D., M. Tsujimoto, Y. Terano, D. W. Scott and C. B. Saper (1993). "Distribution and characterization of tumor necrosis factor-alpha-like immunoreactivity in the murine central nervous system." J Comp Neurol **337**(4): 543-67.

Brickley, S. G., C. Misra, M. H. Mok, M. Mishina and S. G. Cull-Candy (2003). "NR2B and NR2D subunits coassemble in cerebellar Golgi cells to form a distinct NMDA receptor subtype restricted to extrasynaptic sites." J Neurosci **23**(12): 4958-66.

Brothwell, S. L., J. L. Barber, D. T. Monaghan, D. E. Jane, A. J. Gibb and S. Jones (2008). "NR2B- and NR2D-containing synaptic NMDA receptors in developing rat substantia nigra pars compacta dopaminergic neurones." J Physiol **586**(3): 739-50.

Buchsbaum, R. J., B. A. Connolly and L. A. Feig (2002). "Interaction of Rac exchange factors Tiam1 and Ras-GRF1 with a scaffold for the p38 mitogen-activated protein kinase cascade." Mol Cell Biol **22**(12): 4073-85.

Buller, A. L., H. C. Larson, B. E. Schneider, J. A. Beaton, R. A. Morrisett and D. T. Monaghan (1994). "The molecular basis of NMDA receptor subtypes: native receptor diversity is predicted by subunit composition." J Neurosci **14**(9): 5471-84.

Burack, W. R. and A. S. Shaw (2000). "Signal transduction: hanging on a scaffold." Curr Opin Cell Biol **12**(2): 211-6.

Burke, R. E. (2007). "Inhibition of mitogen-activated protein kinase and stimulation of Akt kinase signaling pathways: Two approaches with therapeutic potential in the treatment of neurodegenerative disease." Pharmacol Ther **114**(3): 261-77.

Calabresi, P., E. Saulle, G. A. Marfia, D. Centonze, R. Mulloy, B. Picconi, R. A. Hipskind, F. Conquet and G. Bernardi (2001). "Activation of metabotropic glutamate receptor subtype 1/protein kinase C/mitogen-activated protein kinase pathway is required for postischemic long-term potentiation in the striatum." Mol Pharmacol **60**(4): 808-15.

Calderone, A., T. Jover, K. M. Noh, H. Tanaka, H. Yokota, Y. Lin, S. Y. Grooms, R. Regis, M. V. Bennett and R. S. Zukin (2003). "Ischemic insults derepress the gene silencer REST in neurons destined to die." J Neurosci **23**(6): 2112-21.

Cao, G., R. S. Clark, W. Pei, W. Yin, F. Zhang, F. Y. Sun, S. H. Graham and J. Chen (2003). "Translocation of apoptosis-inducing factor in vulnerable neurons after transient cerebral ischemia and in neuronal cultures after oxygen-glucose deprivation." J Cereb Blood Flow Metab **23**(10): 1137-50.

Cao, G., M. Xiao, F. Sun, X. Xiao, W. Pei, J. Li, S. H. Graham, R. P. Simon and J. Chen (2004a). "Cloning of a novel Apaf-1-interacting protein: a potent suppressor of apoptosis and ischemic neuronal cell death." J Neurosci **24**(27): 6189-201.

Cao, J., M. M. Semenova, V. T. Solovyan, J. Han, E. T. Coffey and M. J. Courtney (2004b). "Distinct requirements for p38alpha and c-Jun N-terminal kinase stress-activated protein kinases in different forms of apoptotic neuronal death." J Biol Chem **279**(34): 35903-13.

Cao, J., J. I. Viholainen, C. Dart, H. K. Warwick, M. L. Leyland and M. J. Courtney (2005). "The PSD95-nNOS interface: a target for inhibition of excitotoxic p38 stress-activated protein kinase activation and cell death." J Cell Biol **168**(1): 117-26.

Carboni, S., A. Hiver, C. Szyndralewiez, P. Gaillard, J. P. Gotteland and P. A. Vitte (2004). "AS601245 (1,3-benzothiazol-2-yl (2-[[2-(3-pyridinyl) ethyl] amino]-4 pyrimidinyl) acetonitrile): a c-Jun NH2-terminal protein kinase inhibitor with neuroprotective properties." J Pharmacol Exp Ther **310**(1): 25-32.

Cardoso, A. L., P. Costa, L. P. de Almeida, S. Simoes, N. Plesnila, C. Culmsee, E. Wagner and M. C. de Lima (2010). "Tf-lipoplex-mediated c-Jun silencing improves neuronal survival following excitotoxic damage in vivo." J Control Release **142**(3): 392-403.

Catarzi, D., V. Colotta and F. Varano (2007). "Competitive AMPA receptor antagonists." Med Res Rev **27**(2): 239-78.

Centeno, C., M. Repici, J. Y. Chatton, B. M. Riederer, C. Bonny, P. Nicod, M. Price, P. G. Clarke, S. Papa, G. Franzoso and T. Borsello (2007). "Role of the JNK pathway in NMDA-mediated excitotoxicity of cortical neurons." Cell Death Differ **14**(2): 240-53.

Cepeda, C. and M. S. Levine (2012). "2B or not 2B: a tail of two NMDA receptor subunits." Neuron **74**(3): 426-8.

Chan, F. K. and E. H. Baehrecke (2012). "RIP3 finds partners in crime." Cell **148**(1-2): 17-8.

Chandler, L. J., G. Sutton, N. R. Dorairaj and D. Norwood (2001). "N-methyl D-aspartate receptor-mediated bidirectional control of extracellular signal-regulated kinase activity in cortical neuronal cultures." J Biol Chem **276**(4): 2627-36.

Chavez-Valdez, R., L. J. Martin, D. L. Flock and F. J. Northington (2012). "Necrostatin-1 attenuates mitochondrial dysfunction in neurons and astrocytes following neonatal hypoxia-ischemia." Neuroscience **219**: 192-203.

Chen, B. S. and K. W. Roche (2007). "Regulation of NMDA receptors by phosphorylation." Neuropharmacology **53**(3): 362-8.

Chen, Z., T. B. Gibson, F. Robinson, L. Silvestro, G. Pearson, B. Xu, A. Wright, C. Vanderbilt and M. H. Cobb (2001). "MAP kinases." Chem Rev **101**(8): 2449-76.

Chi, H., S. P. Barry, R. J. Roth, J. J. Wu, E. A. Jones, A. M. Bennett and R. A. Flavell (2006). "Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses." Proc Natl Acad Sci U S A **103**(7): 2274-9.

Chiariello, M., M. J. Marinissen and J. S. Gutkind (2000). "Multiple mitogen-activated protein kinase signaling pathways connect the cot oncoprotein to the c-jun promoter and to cellular transformation." Mol Cell Biol **20**(5): 1747-58.

Chinnaiyan, A. M. (1999). "The apoptosome: heart and soul of the cell death machine." Neoplasia **1**(1): 5-15.

Cho, Y. S., S. Challa, D. Moquin, R. Genga, T. D. Ray, M. Guildford and F. K. Chan (2009). "Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation." Cell **137**(6): 1112-23.

Choi, D. W. (1987). "Ionic dependence of glutamate neurotoxicity." J Neurosci **7**(2): 369-79.

Choi, D. W. (1988). "Glutamate neurotoxicity and diseases of the nervous system." Neuron **1**(8): 623-34.

Choi, D. W., M. Maulucci-Gedde and A. R. Kriegstein (1987). "Glutamate neurotoxicity in cortical cell culture." J Neurosci **7**(2): 357-68.

Christofferson, D. E., Y. Li, J. Hitomi, W. Zhou, C. Upperman, H. Zhu, S. A. Gerber, S. Gygi and J. Yuan (2012). "A novel role for RIP1 kinase in mediating TNFalpha production." Cell Death Dis **3**: e320.

Chrivia, J. C., R. P. Kwok, N. Lamb, M. Hagiwara, M. R. Montminy and R. H. Goodman (1993). "Phosphorylated CREB binds specifically to the nuclear protein CBP." Nature **365**(6449): 855-9.

Chung, H. J., Y. H. Huang, L. F. Lau and R. L. Huganir (2004). "Regulation of the NMDA receptor complex and trafficking by activity-dependent phosphorylation of the NR2B subunit PDZ ligand." J Neurosci **24**(45): 10248-59.

Coffey, E. T., V. Hongisto, M. Dickens, R. J. Davis and M. J. Courtney (2000). "Dual roles for c-Jun N-terminal kinase in developmental and stress responses in cerebellar granule neurons." J Neurosci **20**(20): 7602-13.

Coffey, E. T., G. Smiciene, V. Hongisto, J. Cao, S. Brecht, T. Herdegen and M. J. Courtney (2002). "c-Jun N-terminal protein kinase (JNK) 2/3 is specifically activated by stress, mediating c-Jun activation, in the presence of constitutive JNK1 activity in cerebellar neurons." J Neurosci **22**(11): 4335-45.

Cook, D. J., L. Teves and M. Tymianski (2012). "Treatment of stroke with a PSD-95 inhibitor in the gyrencephalic primate brain." Nature **483**(7388): 213-7.

Cory, S. and J. M. Adams (2002). "The Bcl2 family: regulators of the cellular life-or-death switch." Nat Rev Cancer **2**(9): 647-56.

Coulthard, L. R., D. E. White, D. L. Jones, M. F. McDermott and S. A. Burchill (2009). "p38(MAPK): stress responses from molecular mechanisms to therapeutics." Trends Mol Med **15**(8): 369-79.

Cui, H., A. Hayashi, H. S. Sun, M. P. Belmares, C. Cobey, T. Phan, J. Schweizer, M. W. Salter, Y. T. Wang, R. A. Tasker, D. Garman, J. Rabinowitz, P. S. Lu and M. Tymianski (2007a). "PDZ protein interactions underlying NMDA receptor-mediated excitotoxicity and neuroprotection by PSD-95 inhibitors." J Neurosci **27**(37): 9901-15.

Cui, J., M. Zhang, Y. Q. Zhang and Z. H. Xu (2007b). "JNK pathway: diseases and therapeutic potential." Acta Pharmacol Sin **28**(5): 601-8.

Culbert, A. A., S. D. Skaper, D. R. Howlett, N. A. Evans, L. Facci, P. E. Soden, Z. M. Seymour, F. Guillot, M. Gaestel and J. C. Richardson (2006). "MAPK-activated protein kinase 2 deficiency in microglia inhibits pro-inflammatory mediator release and resultant neurotoxicity. Relevance to neuroinflammation in a

transgenic mouse model of Alzheimer disease." J Biol Chem **281**(33): 23658-67.

Cull-Candy, S., L. Kelly and M. Farrant (2006). "Regulation of Ca2+-permeable AMPA receptors: synaptic plasticity and beyond." Curr Opin Neurobiol **16**(3): 288-97.

Cull-Candy, S. G. and D. N. Leszkiewicz (2004). "Role of distinct NMDA receptor subtypes at central synapses." Sci STKE **2004**(255): re16.

Culmsee, C., C. Zhu, S. Landshamer, B. Becattini, E. Wagner, M. Pellecchia, K. Blomgren and N. Plesnila (2005). "Apoptosis-inducing factor triggered by poly(ADP-ribose) polymerase and Bid mediates neuronal cell death after oxygen-glucose deprivation and focal cerebral ischemia." J Neurosci **25**(44): 10262-72.

Dahm, R., M. Zeitelhofer, B. Gotze, M. A. Kiebler and P. Macchi (2008). "Visualizing mRNA localization and local protein translation in neurons." Methods Cell Biol **85**: 293-327.

Das, S., J. Cho, I. Lambertz, M. A. Kelliher, A. G. Eliopoulos, K. Du and P. N. Tsichlis (2005). "Tpl2/cot signals activate ERK, JNK, and NF-kappaB in a cell-type and stimulus-specific manner." J Biol Chem **280**(25): 23748-57.

Davis, R. J. (2000). "Signal transduction by the JNK group of MAP kinases." Cell **103**(2): 239-52.

Declercq, W., T. Vanden Berghe and P. Vandenabeele (2009). "RIP kinases at the crossroads of cell death and survival." Cell **138**(2): 229-32.

Degterev, A., J. Hitomi, M. Germscheid, I. L. Ch'en, O. Korkina, X. Teng, D. Abbott, G. D. Cuny, C. Yuan, G. Wagner, S. M. Hedrick, S. A. Gerber, A. Lugovskoy and J. Yuan (2008). "Identification of RIP1 kinase as a specific cellular target of necrostatins." Nat Chem Biol **4**(5): 313-21.

Degterev, A., Z. Huang, M. Boyce, Y. Li, P. Jagtap, N. Mizushima, G. D. Cuny, T. J. Mitchison, M. A. Moskowitz and J. Yuan (2005). "Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury." Nat Chem Biol **1**(2): 112-9.

Del Villar, K. and C. A. Miller (2004). "Down-regulation of DENN/MADD, a TNF receptor binding protein, correlates with neuronal cell death in Alzheimer's disease brain and hippocampal neurons." Proc Natl Acad Sci U S A **101**(12): 4210-5.

Derijard, B., M. Hibi, I. H. Wu, T. Barrett, B. Su, T. Deng, M. Karin and R. J. Davis (1994). "JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain." Cell **76**(6): 1025-37.

Deupree, D. L., X. W. Tang, M. Yarom, E. Dickman, R. D. Kirch, J. V. Schloss and J. Y. Wu (1996). "Studies of NMDA- and non-NMDA-mediated neurotoxicity in cultured neurons." Neurochem Int **29**(3): 255-61.

Devin, A., Y. Lin and Z. G. Liu (2003). "The role of the death-domain kinase RIP in tumour-necrosis-factor-induced activation of mitogen-activated protein kinases." EMBO Rep **4**(6): 623-7.

Diarra, D., M. Stolina, K. Polzer, J. Zwerina, M. S. Ominsky, D. Dwyer, A. Korb, J. Smolen, M. Hoffmann, C. Scheinecker, D. van der Heide, R. Landewe, D. Lacey, W. G. Richards and G. Schett (2007). "Dickkopf-1 is a master regulator of joint remodeling." Nat Med **13**(2): 156-63.

Dick, O. and H. Bading (2010). "Synaptic activity and nuclear calcium signaling protect hippocampal neurons from death signal-associated nuclear translocation of FoxO3a induced by extrasynaptic N-methyl-D-aspartate receptors." J Biol Chem **285**(25): 19354-61.

Dickens, L. S., I. R. Powley, M. A. Hughes and M. MacFarlane (2012). "The 'complexities' of life and death: death receptor signalling platforms." Exp Cell Res **318**(11): 1269-77.

Dieterich, D. C., A. Karpova, M. Mikhaylova, I. Zdobnova, I. Konig, M. Landwehr, M. Kreutz, K. H. Smalla, K. Richter, P. Landgraf, C. Reissner, T. M. Boeckers, W. Zuschratter, C. Spilker, C. I. Seidenbecher, C. C. Garner, E. D. Gundelfinger and M. R. Kreutz (2008). "Caldendrin-Jacob: a protein liaison that couples NMDA receptor signalling to the nucleus." PLoS Biol **6**(2): e34.

Donovan, N., E. B. Becker, Y. Konishi and A. Bonni (2002). "JNK phosphorylation and activation of BAD couples the stress-activated signaling pathway to the cell death machinery." J Biol Chem **277**(43): 40944-9.

Du, C., M. Fang, Y. Li, L. Li and X. Wang (2000). "Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition." Cell **102**(1): 33-42.

Duprez, L., M. J. Bertrand, T. Vanden Berghe, Y. Dondelinger, N. Festjens and P. Vandenabeele (2012). "Intermediate domain of receptor-interacting protein kinase 1 (RIPK1) determines switch between necroptosis and RIPK1 kinase-dependent apoptosis." J Biol Chem **287**(18): 14863-72.

Ea, C. K., L. Deng, Z. P. Xia, G. Pineda and Z. J. Chen (2006). "Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO." Mol Cell **22**: 245-57.

Ehlers, M. D. (2000). "Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting." Neuron **28**(2): 511-25.

Eilers, A., J. Whitfield, C. Babij, L. L. Rubin and J. Ham (1998). "Role of the Jun kinase pathway in the regulation of c-Jun expression and apoptosis in sympathetic neurons." J Neurosci **18**(5): 1713-24.

Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu and S. Nagata (1998). "A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD." Nature **391**(6662): 43-50.

Enesa, K., M. Zakkar, H. Chaudhury, A. Luong le, L. Rawlinson, J. C. Mason, D. O. Haskard, J. L. Dean and P. C. Evans (2008). "NF-kappaB suppression by the deubiquitinating enzyme Cezanne: a novel negative feedback loop in pro-inflammatory signaling." J Biol Chem **283**(11): 7036-45.

Enslen, H., P. Sun, D. Brickey, S. H. Soderling, E. Klamo and T. R. Soderling (1994). "Characterization of Ca2+/calmodulin-dependent protein kinase IV. Role in transcriptional regulation." J Biol Chem **269**(22): 15520-7.

Esposti, M. D. (2002). "The roles of Bid." Apoptosis **7**(5): 433-40.

Fadok, V. A., A. de Cathelineau, D. L. Daleke, P. M. Henson and D. L. Bratton (2001). "Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts." J Biol Chem **276**(2): 1071-7.

Farinelli, M., F. D. Heitz, B. F. Grewe, S. K. Tyagarajan, F. Helmchen and I. M. Mansuy (2012). "Selective regulation of NR2B by protein phosphatase-1 for the control of the NMDA receptor in neuroprotection." PLoS One **7**(3): e34047.

Feltrin, D., L. Fusco, H. Witte, F. Moretti, K. Martin, M. Letzelter, E. Fluri, P. Scheiffele and O. Pertz (2012). "Growth cone MKK7 mRNA targeting regulates MAP1b-dependent microtubule bundling to control neurite elongation." PLoS Biol **10**(12): e1001439.

Feng, S., L. Ma, Y. Yang and M. Wu (2006). "Truncated RIP3 (tRIP3) acts upstream of FADD to induce apoptosis in the human hepatocellular carcinoma cell line QGY-7703." Biochem Biophys Res Commun **347**(3): 558-65.

Feng, S., Y. Yang, Y. Mei, L. Ma, D. E. Zhu, N. Hoti, M. Castanares and M. Wu (2007). "Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain." Cell Signal **19**(10): 2056-67.

Feoktistova, M., P. Geserick, B. Kellert, D. P. Dimitrova, C. Langlais, M. Hupe, K. Cain, M. MacFarlane, G. Hacker and M. Leverkus (2011). "cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms." Mol Cell **43**(3): 449-63.

Ferreira, J. S., A. Rooyakkers, K. She, L. Ribeiro, A. L. Carvalho and A. M. Craig (2011). "Activity and protein kinase C regulate synaptic accumulation of N-

methyl-D-aspartate (NMDA) receptors independently of GluN1 splice variant." J Biol Chem **286**(32): 28331-42.

Ferrer, I., J. Ballabriga and E. Pozas (1997). "Transient forebrain ischemia in the adult gerbil is associated with a complex c-Jun response." Neuroreport **8**(11): 2483-7.

Ferrer, I., T. Gomez-Isla, B. Puig, M. Freixes, E. Ribe, E. Dalfo and J. Avila (2005). "Current advances on different kinases involved in tau phosphorylation, and implications in Alzheimer's disease and tauopathies." Curr Alzheimer Res **2**(1): 3-18.

Fiers, W., R. Beyaert, E. Boone, S. Cornelis, W. Declercq, E. Decoster, G. Denecker, B. Depuydt, D. De Valck, G. De Wilde, V. Goossens, J. Grooten, G. Haegeman, K. Heyninck, L. Penning, S. Plaisance, K. Vancompernolle, W. Van Criekinge, P. Vandenabeele, W. Vanden Berghe, M. Van de Craen, V. Vandevoorde and D. Vercammen (1995). "TNF-induced intracellular signaling leading to gene induction or to cytotoxicity by necrosis or by apoptosis." J Inflamm **47**(1-2): 67-75.

Finkel, T. and N. J. Holbrook (2000). "Oxidants, oxidative stress and the biology of ageing." Nature **408**(6809): 239-47.

Flynn, R. W., R. S. MacWalter and A. S. Doney (2008). "The cost of cerebral ischaemia." Neuropharmacology **55**(3): 250-6.

Fonarow, G. C., E. E. Smith, J. L. Saver, M. J. Reeves, D. L. Bhatt, M. V. Grau-Sepulveda, D. M. Olson, A. F. Hernandez, E. D. Peterson and L. H. Schwamm (2011). "Timeliness of tissue-type plasminogen activator therapy in acute ischemic stroke: patient characteristics, hospital factors, and outcomes associated with door-to-needle times within 60 minutes." Circulation **123**(7): 750-8.

Fong, D. K., A. Rao, F. T. Crump and A. M. Craig (2002). "Rapid synaptic remodeling by protein kinase C: reciprocal translocation of NMDA receptors and calcium/calmodulin-dependent kinase II." J Neurosci **22**(6): 2153-64.

Forder, J. P. and M. Tymianski (2009). "Postsynaptic mechanisms of excitotoxicity: Involvement of postsynaptic density proteins, radicals, and oxidant molecules." Neuroscience **158**(1): 293-300.

Fricker, M., A. Vilalta, A. M. Tolkovsky and G. C. Brown (2013). "Caspase inhibitors protect neurons by enabling selective necroptosis of inflamed microglia." J Biol Chem **288**(13): 9145-52.

Friedman, L. K. (2006). "Calcium: a role for neuroprotection and sustained adaptation." Mol Interv **6**(6): 315-29.

Frizelle, P. A., P. E. Chen and D. J. Wyllie (2006). "Equilibrium constants for (R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5 -yl)-methyl]-phosphonic acid (NVP-AAM077) acting at recombinant NR1/NR2A and NR1/NR2B N-methyl-D-aspartate receptors: Implications for studies of synaptic transmission." Mol Pharmacol **70**(3): 1022-32.

Fukaya, M., A. Kato, C. Lovett, S. Tonegawa and M. Watanabe (2003). "Retention of NMDA receptor NR2 subunits in the lumen of endoplasmic reticulum in targeted NR1 knockout mice." Proc Natl Acad Sci U S A **100**(8): 4855-60.

Fukui, M., H. J. Choi and B. T. Zhu (2012). "Rapid generation of mitochondrial superoxide induces mitochondrion-dependent but caspase-independent cell death in hippocampal neuronal cells that morphologically resembles necroptosis." Toxicol Appl Pharmacol **262**(2): 156-66.

Furukawa, H. and E. Gouaux (2003). "Mechanisms of activation, inhibition and specificity: crystal structures of the NMDA receptor NR1 ligand-binding core." EMBO J **22**(12): 2873-85.

Gaither, A., D. Porter, Y. Yao, J. Borawski, G. Yang, J. Donovan, D. Sage, J. Slisz, M. Tran, C. Straub, T. Ramsey, V. Iourgenko, A. Huang, Y. Chen, R. Schlegel, M. Labow, S. Fawell, W. R. Sellers and L. Zawel (2007). "A Smac mimetic rescue screen reveals roles for inhibitor of apoptosis proteins in tumor necrosis factor-alpha signaling." Cancer Res **67**(24): 11493-8.

Gamble, J. R., J. M. Harlan, S. J. Klebanoff and M. A. Vadas (1985). "Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor." Proc Natl Acad Sci U S A **82**(24): 8667-71.

Gao, Y., A. P. Signore, W. Yin, G. Cao, X. M. Yin, F. Sun, Y. Luo, S. H. Graham and J. Chen (2005). "Neuroprotection against focal ischemic brain injury by inhibition of c-Jun N-terminal kinase and attenuation of the mitochondrial apoptosis-signaling pathway." J Cereb Blood Flow Metab **25**(6): 694-712.

Gardoni, F., C. Bellone, F. Cattabeni and M. Di Luca (2001). "Protein kinase C activation modulates alpha-calmodulin kinase II binding to NR2A subunit of N-methyl-D-aspartate receptor complex." J Biol Chem **276**(10): 7609-13.

Gardoni, F., E. Marcello and M. Di Luca (2009). "Postsynaptic density-membrane associated guanylate kinase proteins (PSD-MAGUKs) and their role in CNS disorders." Neuroscience **158**(1): 324-33.

Gardoni, F., D. Mauceri, C. Fiorentini, C. Bellone, C. Missale, F. Cattabeni and M. Di Luca (2003). "CaMKII-dependent phosphorylation regulates SAP97/NR2A interaction." J Biol Chem **278**(45): 44745-52.

Garrido, C., L. Galluzzi, M. Brunet, P. E. Puig, C. Didelot and G. Kroemer (2006). "Mechanisms of cytochrome c release from mitochondria." Cell Death Differ **13**(9): 1423-33.

Ge, B., H. Gram, F. Di Padova, B. Huang, L. New, R. J. Ulevitch, Y. Luo and J. Han (2002). "MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha." Science **295**(5558): 1291-4.

Gee, C. E., P. Benquet, O. Raineteau, L. Rietschin, S. W. Kirbach and U. Gerber (2006). "NMDA receptors and the differential ischemic vulnerability of hippocampal neurons." Eur J Neurosci **23**(10): 2595-603.

Geserick, P., M. Hupe, M. Moulin, W. W. Wong, M. Feoktistova, B. Kellert, H. Gollnick, J. Silke and M. Leverkus (2009). "Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment." J Cell Biol **187**(7): 1037-54.

Ghosh, A. and M. E. Greenberg (1995). "Calcium signaling in neurons: molecular mechanisms and cellular consequences." Science **268**(5208): 239-47.

Gilley, J., P. J. Coffer and J. Ham (2003). "FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons." J Cell Biol **162**(4): 613-22.

Goldberg, M. P. and D. W. Choi (1993). "Combined oxygen and glucose deprivation in cortical cell culture: calcium-dependent and calcium-independent mechanisms of neuronal injury." J Neurosci **13**(8): 3510-24.

Golks, A., D. Brenner, C. Fritsch, P. H. Krammer and I. N. Lavrik (2005). "c-FLIPR, a new regulator of death receptor-induced apoptosis." J Biol Chem **280**(15): 14507-13.

Gorter, J. A., J. J. Petrozzino, E. M. Aronica, D. M. Rosenbaum, T. Opitz, M. V. Bennett, J. A. Connor and R. S. Zukin (1997). "Global ischemia induces downregulation of Glur2 mRNA and increases AMPA receptor-mediated Ca2+ influx in hippocampal CA1 neurons of gerbil." J Neurosci **17**(16): 6179-88.

Graham, D., G. Darles and S. Z. Langer (1992). "The neuroprotective properties of ifenprodil, a novel NMDA receptor antagonist, in neuronal cell culture toxicity studies." Eur J Pharmacol **226**(4): 373-6.

Greger, I. H., E. B. Ziff and A. C. Penn (2007). "Molecular determinants of AMPA receptor subunit assembly." Trends Neurosci **30**(8): 407-16.

Groc, L. and D. Choquet (2006). "AMPA and NMDA glutamate receptor trafficking: multiple roads for reaching and leaving the synapse." Cell Tissue Res **326**(2): 423-38.

Groc, L., M. Heine, L. Cognet, K. Brickley, F. A. Stephenson, B. Lounis and D. Choquet (2004). "Differential activity-dependent regulation of the lateral mobilities of AMPA and NMDA receptors." Nat Neurosci **7**(7): 695-6.

Groc, L., M. Heine, S. L. Cousins, F. A. Stephenson, B. Lounis, L. Cognet and D. Choquet (2006). "NMDA receptor surface mobility depends on NR2A-2B subunits." Proc Natl Acad Sci U S A **103**(49): 18769-74.

Gruen, J. R. and S. M. Weissman (1997). "Evolving views of the major histocompatibility complex." Blood **90**(11): 4252-65.

Gunther, C., E. Martini, N. Wittkopf, K. Amann, B. Weigmann, H. Neumann, M. J. Waldner, S. M. Hedrick, S. Tenzer, M. F. Neurath and C. Becker (2011). "Caspase-8 regulates TNF-alpha-induced epithelial necroptosis and terminal ileitis." Nature **477**(7364): 335-9.

Guo, G. and N. R. Bhat (2007). "p38alpha MAP kinase mediates hypoxia-induced motor neuron cell death: a potential target of minocycline's neuroprotective action." Neurochem Res **32**(12): 2160-6.

Gupta, S., T. Barrett, A. J. Whitmarsh, J. Cavanagh, H. K. Sluss, B. Derijard and R. J. Davis (1996). "Selective interaction of JNK protein kinase isoforms with transcription factors." EMBO J **15**(11): 2760-70.

Gwag, B. J., D. Lobner, J. Y. Koh, M. B. Wie and D. W. Choi (1995). "Blockade of glutamate receptors unmasks neuronal apoptosis after oxygen-glucose deprivation in vitro." Neuroscience **68**(3): 615-9.

Haas, T. L., C. H. Emmerich, B. Gerlach, A. C. Schmukle, S. M. Cordier, E. Rieser, R. Feltham, J. Vince, U. Warnken, T. Wenger, R. Koschny, D. Komander, J. Silke and H. Walczak (2009). "Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction." Mol Cell **36**(5): 831-44.

Hagemann, D., J. Troppmair and U. R. Rapp (1999). "Cot protooncoprotein activates the dual specificity kinases MEK-1 and SEK-1 and induces differentiation of PC12 cells." Oncogene **18**(7): 1391-400.

Ham, J., C. Babij, J. Whitfield, C. M. Pfarr, D. Lallemand, M. Yaniv and L. L. Rubin (1995). "A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death." Neuron **14**(5): 927-39.

Han, J., C. Q. Zhong and D. W. Zhang (2011). "Programmed necrosis: backup to and competitor with apoptosis in the immune system." Nat Immunol **12**(12): 1143-9.

Hansen, H. H., T. Briem, M. Dzietko, M. Sifringer, A. Voss, W. Rzeski, B. Zdzisinska, F. Thor, R. Heumann, A. Stepulak, P. Bittigau and C. Ikonomidou (2004).

"Mechanisms leading to disseminated apoptosis following NMDA receptor blockade in the developing rat brain." Neurobiol Dis **16**(2): 440-53.

Hansen, K. B., H. Yuan and S. F. Traynelis (2007). "Structural aspects of AMPA receptor activation, desensitization and deactivation." Curr Opin Neurobiol **17**(3): 281-8.

Hardingham, G. E. and H. Bading (2002). "Coupling of extrasynaptic NMDA receptors to a CREB shut-off pathway is developmentally regulated." Biochim Biophys Acta **1600**(1-2): 148-53.

Hardingham, G. E. and H. Bading (2010). "Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders." Nat Rev Neurosci **11**(10): 682-96.

Hardingham, G. E., S. Chawla, C. M. Johnson and H. Bading (1997). "Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression." Nature **385**(6613): 260-5.

Hardingham, G. E., Y. Fukunaga and H. Bading (2002). "Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways." Nat Neurosci **5**(5): 405-14.

Harper, S. J. and P. LoGrasso (2001). "Signalling for survival and death in neurones: the role of stress-activated kinases, JNK and p38." Cell Signal **13**(5): 299-310.

Harper, S. J. and N. Wilkie (2003). "MAPKs: new targets for neurodegeneration." Expert Opin Ther Targets **7**(2): 187-200.

Harris, A. Z. and D. L. Pettit (2007). "Extrasynaptic and synaptic NMDA receptors form stable and uniform pools in rat hippocampal slices." J Physiol **584**(Pt 2): 509-19.

Harris, C. A. and E. M. Johnson, Jr. (2001). "BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons." J Biol Chem **276**(41): 37754-60.

Hazell, A. S. (2007). "Excitotoxic mechanisms in stroke: an update of concepts and treatment strategies." Neurochem Int **50**(7-8): 941-53.

He, K. L. and A. T. Ting (2002). "A20 inhibits tumor necrosis factor (TNF) alpha-induced apoptosis by disrupting recruitment of TRADD and RIP to the TNF receptor 1 complex in Jurkat T cells." Mol Cell Biol **22**(17): 6034-45.

He, S., L. Wang, L. Miao, T. Wang, F. Du, L. Zhao and X. Wang (2009). "Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha." Cell **137**(6): 1100-11.

Herdegen, T., F. X. Claret, T. Kallunki, A. Martin-Villalba, C. Winter, T. Hunter and M. Karin (1998). "Lasting N-terminal phosphorylation of c-Jun and activation of c-Jun N-terminal kinases after neuronal injury." J Neurosci **18**(14): 5124-35.

Herdegen, T. and V. Waetzig (2001). "AP-1 proteins in the adult brain: facts and fiction about effectors of neuroprotection and neurodegeneration." Oncogene **20**(19): 2424-37.

Hess, J., P. Angel and M. Schorpp-Kistner (2004). "AP-1 subunits: quarrel and harmony among siblings." J Cell Sci **117**(Pt 25): 5965-73.

Hetman, M. and G. Kharebava (2006). "Survival signaling pathways activated by NMDA receptors." Curr Top Med Chem **6**(8): 787-99.

Hill, M. M., C. Adrain, P. J. Duriez, E. M. Creagh and S. J. Martin (2004). "Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes." EMBO J **23**(10): 2134-45.

Hirokawa, N. and R. Takemura (2004). "Molecular motors in neuronal development, intracellular transport and diseases." Curr Opin Neurobiol **14**(5): 564-73.

Hirosumi, J., G. Tuncman, L. Chang, C. Z. Gorgun, K. T. Uysal, K. Maeda, M. Karin and G. S. Hotamisligil (2002). "A central role for JNK in obesity and insulin resistance." Nature **420**(6913): 333-6.

Hirt, L., J. Badaut, J. Thevenet, C. Granziera, L. Regli, F. Maurer, C. Bonny and J. Bogousslavsky (2004). "D-JNKI1, a cell-penetrating c-Jun-N-terminal kinase inhibitor, protects against cell death in severe cerebral ischemia." Stroke **35**(7): 1738-43.

Hitomi, J., D. E. Christofferson, A. Ng, J. Yao, A. Degterev, R. J. Xavier and J. Yuan (2008). "Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway." Cell **135**(7): 1311-23.

Holler, N., R. Zaru, O. Micheau, M. Thome, A. Attinger, S. Valitutti, J. L. Bodmer, P. Schneider, B. Seed and J. Tschopp (2000). "Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule." Nat Immunol **1**(6): 489-95.

Hollmann, M. and S. Heinemann (1994). "Cloned glutamate receptors." Annu Rev Neurosci **17**: 31-108.

Hou, S. T., Y. Tu, A. M. Buchan, Z. Huang, E. Preston, I. Rasquinha, G. S. Robertson and J. P. MacManus (1997). "Increases in DNA lesions and the DNA damage indicator Gadd45 following transient cerebral ischemia." Biochem Cell Biol **75**(4): 383-92.

Hu, J. H., T. Chen, Z. H. Zhuang, L. Kong, M. C. Yu, Y. Liu, J. W. Zang and B. X. Ge (2007). "Feedback control of MKP-1 expression by p38." Cell Signal **19**(2): 393-400.

Hu, S. Q., J. Zhu, D. S. Pei, Y. Y. Zong, J. Z. Yan, X. Y. Hou and G. Y. Zhang (2009). "Overexpression of the PDZ1 domain of PSD-95 diminishes ischemic brain

injury via inhibition of the GluR6.PSD-95.MLK3 pathway." J Neurosci Res **87**(16): 3626-38.

Huh, K. H. and R. J. Wenthold (1999). "Turnover analysis of glutamate receptors identifies a rapidly degraded pool of the N-methyl-D-aspartate receptor subunit, NR1, in cultured cerebellar granule cells." J Biol Chem **274**(1): 151-7.

Hunot, S., M. Vila, P. Teismann, R. J. Davis, E. C. Hirsch, S. Przedborski, P. Rakic and R. A. Flavell (2004). "JNK-mediated induction of cyclooxygenase 2 is required for neurodegeneration in a mouse model of Parkinson's disease." Proc Natl Acad Sci U S A **101**(2): 665-70.

Hurtado, O., A. Cardenas, I. Lizasoain, L. Bosca, J. C. Leza, P. Lorenzo and M. A. Moro (2001). "Up-regulation of TNF-alpha convertase (TACE/ADAM17) after oxygen-glucose deprivation in rat forebrain slices." Neuropharmacology **40**(8): 1094-102.

Hurtado, O., I. Lizasoain, P. Fernandez-Tome, A. Alvarez-Barrientos, J. C. Leza, P. Lorenzo and M. A. Moro (2002). "TACE/ADAM17-TNF-alpha pathway in rat cortical cultures after exposure to oxygen-glucose deprivation or glutamate." J Cereb Blood Flow Metab **22**(5): 576-85.

Iadecola, C. and J. Anrather (2011a). "The immunology of stroke: from mechanisms to translation." Nat Med **17**(7): 796-808.

Iadecola, C. and J. Anrather (2011b). "Stroke research at a crossroad: asking the brain for directions." Nat Neurosci **14**(11): 1363-8.

Igney, F. H. and P. H. Krammer (2002). "Death and anti-death: tumour resistance to apoptosis." Nat Rev Cancer **2**(4): 277-88.

Ikner, A. and A. Ashkenazi (2011). "TWEAK induces apoptosis through a death-signaling complex comprising receptor-interacting protein 1 (RIP1), Fas-associated death domain (FADD), and caspase-8." J Biol Chem **286**(24): 21546-54.

Irrinki, K. M., K. Mallilankaraman, R. J. Thapa, H. C. Chandramoorthy, F. J. Smith, N. R. Jog, R. K. Gandhirajan, S. G. Kelsen, S. R. Houser, M. J. May, S. Balachandran and M. Madesh (2011). "Requirement of FADD, NEMO, and BAX/BAK for aberrant mitochondrial function in tumor necrosis factor alpha-induced necrosis." Mol Cell Biol **31**(18): 3745-58.

Ivanov, A., C. Pellegrino, S. Rama, I. Dumalska, Y. Salyha, Y. Ben-Ari and I. Medina (2006). "Opposing role of synaptic and extrasynaptic NMDA receptors in regulation of the extracellular signal-regulated kinases (ERK) activity in cultured rat hippocampal neurons." J Physiol **572**(Pt 3): 789-98.

Jaeschke, A. and R. J. Davis (2007). "Metabolic stress signaling mediated by mixed-lineage kinases." Mol Cell **27**(3): 498-508.

Jaeschke, A., M. Rincon, B. Doran, J. Reilly, D. Neuberg, D. L. Greiner, L. D. Shultz, A. A. Rossini, R. A. Flavell and R. J. Davis (2005). "Disruption of the Jnk2 (Mapk9) gene reduces destructive insulitis and diabetes in a mouse model of type I diabetes." Proc Natl Acad Sci U S A **102**(19): 6931-5.

Jia, J., X. Wang, H. Li, S. Han, P. Zu and J. Li (2007). "Activations of nPKCepsilon and ERK1/2 were involved in oxygen-glucose deprivation-induced neuroprotection via NMDA receptors in hippocampal slices of mice." J Neurosurg Anesthesiol **19**(1): 18-24.

Jo, J., G. H. Son, B. L. Winters, M. J. Kim, D. J. Whitcomb, B. A. Dickinson, Y. B. Lee, K. Futai, M. Amici, M. Sheng, G. L. Collingridge and K. Cho (2010). "Muscarinic receptors induce LTD of NMDAR EPSCs via a mechanism involving hippocalcin, AP2 and PSD-95." Nat Neurosci **13**(10): 1216-24.

Johansen, C., K. Kragballe, M. Westergaard, J. Henningsen, K. Kristiansen and L. Iversen (2005). "The mitogen-activated protein kinases p38 and ERK1/2 are increased in lesional psoriatic skin." Br J Dermatol **152**(1): 37-42.

Johnson, G. L. and R. Lapadat (2002). "Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases." Science **298**(5600): 1911-2.

Johnson, G. V. and C. D. Bailey (2003). "The p38 MAP kinase signaling pathway in Alzheimer's disease." Exp Neurol **183**(2): 263-8.

Johnson, J. W. and P. Ascher (1987). "Glycine potentiates the NMDA response in cultured mouse brain neurons." Nature **325**(6104): 529-31.

Joza, N., S. A. Susin, E. Daugas, W. L. Stanford, S. K. Cho, C. Y. Li, T. Sasaki, A. J. Elia, H. Y. Cheng, L. Ravagnan, K. F. Ferri, N. Zamzami, A. Wakeham, R. Hakem, H. Yoshida, Y. Y. Kong, T. W. Mak, J. C. Zuniga-Pflucker, G. Kroemer and J. M. Penninger (2001). "Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death." Nature **410**(6828): 549-54.

Kaiser, W. J., J. W. Upton, A. B. Long, D. Livingston-Rosanoff, L. P. Daley-Bauer, R. Hakem, T. Caspary and E. S. Mocarski (2011). "RIP3 mediates the embryonic lethality of caspase-8-deficient mice." Nature **471**(7338): 368-72.

Kalai, M., G. Van Loo, T. Vanden Berghe, A. Meeus, W. Burm, X. Saelens and P. Vandenabeele (2002). "Tipping the balance between necrosis and apoptosis in human and murine cells treated with interferon and dsRNA." Cell Death Differ **9**(9): 981-94.

Kalda, A. and A. Zharkovsky (1999). "Metabotropic glutamate receptor agonists protect from oxygen-glucose deprivation- and colchicine-induced apoptosis in primary cultures of cerebellar granule cells." Neuroscience **92**(1): 7-14.

Kamata, H., S. Honda, S. Maeda, L. Chang, H. Hirata and M. Karin (2005). "Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases." Cell **120**(5): 649-61.

Kanayama, A., R. B. Seth, L. Sun, C. K. Ea, M. Hong, A. Shaito, Y. H. Chiu, L. Deng and Z. J. Chen (2004). "TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains." Mol Cell **15**(4): 535-48.

Kanduc, D., A. Mittelman, R. Serpico, E. Sinigaglia, A. A. Sinha, C. Natale, R. Santacroce, M. G. Di Corcia, A. Lucchese, L. Dini, P. Pani, S. Santacroce, S. Simone, R. Bucci and E. Farber (2002). "Cell death: apoptosis versus necrosis (review)." Int J Oncol **21**(1): 165-70.

Kaneto, H., Y. Nakatani, D. Kawamori, T. Miyatsuka, T. A. Matsuoka, M. Matsuhisa and Y. Yamasaki (2006). "Role of oxidative stress, endoplasmic reticulum stress, and c-Jun N-terminal kinase in pancreatic beta-cell dysfunction and insulin resistance." Int J Biochem Cell Biol **38**(5-6): 782-93.

Kapin, M. A., R. Doshi, B. Scatton, L. M. DeSantis and M. L. Chandler (1999). "Neuroprotective effects of eliprodil in retinal excitotoxicity and ischemia." Invest Ophthalmol Vis Sci **40**(6): 1177-82.

Karakas, E., N. Simorowski and H. Furukawa (2011). "Subunit arrangement and phenylethanolamine binding in GluN1/GluN2B NMDA receptors." Nature **475**(7355): 249-53.

Karin, M. and Y. Ben-Neriah (2000). "Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity." Annu Rev Immunol **18**: 621-63.

Karpova, A., M. Mikhaylova, S. Bera, J. Bar, P. P. Reddy, T. Behnisch, V. Rankovic, C. Spilker, P. Bethge, J. Sahin, R. Kaushik, W. Zuschratter, T. Kahne, M. Naumann, E. D. Gundelfinger and M. R. Kreutz (2013). "Encoding and transducing the synaptic or extrasynaptic origin of NMDA receptor signals to the nucleus." Cell **152**(5): 1119-33.

Kataoka, T., M. Schroter, M. Hahne, P. Schneider, M. Irmler, M. Thome, C. J. Froelich and J. Tschopp (1998). "FLIP prevents apoptosis induced by death receptors but not by perforin/granzyme B, chemotherapeutic drugs, and gamma irradiation." J Immunol **161**(8): 3936-42.

Kaufmann, T., A. Strasser and P. J. Jost (2012). "Fas death receptor signalling: roles of Bid and XIAP." Cell Death Differ **19**(1): 42-50.

Kavuri, S. M., P. Geserick, D. Berg, D. P. Dimitrova, M. Feoktistova, D. Siegmund, H. Gollnick, M. Neumann, H. Wajant and M. Leverkus (2011). "Cellular FLICE-inhibitory protein (cFLIP) isoforms block CD95- and TRAIL death receptor-induced gene induction irrespective of processing of caspase-8 or cFLIP in the death-inducing signaling complex." J Biol Chem **286**(19): 16631-46.

Kawasaki, H., T. Morooka, S. Shimohama, J. Kimura, T. Hirano, Y. Gotoh and E. Nishida (1997). "Activation and involvement of p38 mitogen-activated protein kinase in glutamate-induced apoptosis in rat cerebellar granule cells." J Biol Chem **272**(30): 18518-21.

Kelkar, N., C. L. Standen and R. J. Davis (2005). "Role of the JIP4 scaffold protein in the regulation of mitogen-activated protein kinase signaling pathways." Mol Cell Biol **25**(7): 2733-43.

Kelliher, M. A., S. Grimm, Y. Ishida, F. Kuo, B. Z. Stanger and P. Leder (1998). "The death domain kinase RIP mediates the TNF-induced NF-kappaB signal." Immunity **8**(3): 297-303.

Kenchappa, R. S., C. Tep, Z. Korade, S. Urra, F. C. Bronfman, S. O. Yoon and B. D. Carter (2010). "p75 neurotrophin receptor-mediated apoptosis in sympathetic neurons involves a biphasic activation of JNK and up-regulation of tumor necrosis factor-alpha-converting enzyme/ADAM17." J Biol Chem **285**(26): 20358-68.

Kennedy, N. J., G. Martin, A. G. Ehrhardt, J. Cavanagh-Kyros, C. Y. Kuan, P. Rakic, R. A. Flavell, S. N. Treistman and R. J. Davis (2007). "Requirement of JIP scaffold proteins for NMDA-mediated signal transduction." Genes Dev **21**(18): 2336-46.

Kew, J. N., J. G. Richards, V. Mutel and J. A. Kemp (1998). "Developmental changes in NMDA receptor glycine affinity and ifenprodil sensitivity reveal three distinct populations of NMDA receptors in individual rat cortical neurons." J Neurosci **18**(6): 1935-43.

Keyse, S. M. (2000). "Protein phosphatases and the regulation of mitogen-activated protein kinase signalling." Curr Opin Cell Biol **12**(2): 186-92.

Kharbanda, S., S. Saxena, K. Yoshida, P. Pandey, M. Kaneki, Q. Wang, K. Cheng, Y. N. Chen, A. Campbell, T. Sudha, Z. M. Yuan, J. Narula, R. Weichselbaum, C. Nalin and D. Kufe (2000). "Translocation of SAPK/JNK to mitochondria and interaction with Bcl-x(L) in response to DNA damage." J Biol Chem **275**(1): 322-7.

Kim, E. and M. Sheng (2004). "PDZ domain proteins of synapses." Nat Rev Neurosci **5**(10): 771-81.

Kim, J. Y., Y. J. Kim, S. Lee and J. H. Park (2011). "BNip3 is a mediator of TNF-induced necrotic cell death." Apoptosis **16**(2): 114-26.

Kim, M. J., A. W. Dunah, Y. T. Wang and M. Sheng (2005). "Differential roles of NR2A- and NR2B-containing NMDA receptors in Ras-ERK signaling and AMPA receptor trafficking." Neuron **46**(5): 745-60.

Kirino, T. (1982). "Delayed neuronal death in the gerbil hippocampus following ischemia." Brain Res **239**(1): 57-69.

Kischkel, F. C., S. Hellbardt, I. Behrmann, M. Germer, M. Pawlita, P. H. Krammer and M. E. Peter (1995). "Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor." EMBO J **14**(22): 5579-88.

Korb, A., M. Tohidast-Akrad, E. Cetin, R. Axmann, J. Smolen and G. Schett (2006). "Differential tissue expression and activation of p38 MAPK alpha, beta, gamma, and delta isoforms in rheumatoid arthritis." Arthritis Rheum **54**(9): 2745-56.

Kothakota, S., T. Azuma, C. Reinhard, A. Klippel, J. Tang, K. Chu, T. J. McGarry, M. W. Kirschner, K. Koths, D. J. Kwiatkowski and L. T. Williams (1997). "Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis." Science **278**(5336): 294-8.

Koubi, D., H. Jiang, L. Zhang, W. Tang, J. Kuo, A. I. Rodriguez, T. J. Hunter, M. D. Seidman, G. B. Corcoran and R. A. Levine (2005). "Role of Bcl-2 family of proteins in mediating apoptotic death of PC12 cells exposed to oxygen and glucose deprivation." Neurochem Int **46**(1): 73-81.

Kristiansen, M., R. Hughes, P. Patel, T. S. Jacques, A. R. Clark and J. Ham (2010). "Mkp1 is a c-Jun target gene that antagonizes JNK-dependent apoptosis in sympathetic neurons." J Neurosci **30**(32): 10820-32.

Krueger, A., I. Schmitz, S. Baumann, P. H. Krammer and S. Kirchhoff (2001). "Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex." J Biol Chem **276**(23): 20633-40.

Krysko, O., L. De Ridder and M. Cornelissen (2004). "Phosphatidylserine exposure during early primary necrosis (oncosis) in JB6 cells as evidenced by immunogold labeling technique." Apoptosis **9**(4): 495-500.

Kuan, C. Y., A. J. Whitmarsh, D. D. Yang, G. Liao, A. J. Schloemer, C. Dong, J. Bao, K. J. Banasiak, G. G. Haddad, R. A. Flavell, R. J. Davis and P. Rakic (2003). "A critical role of neural-specific JNK3 for ischemic apoptosis." Proc Natl Acad Sci U S A **100**(25): 15184-9.

Kukekov, N. V., Z. Xu and L. A. Greene (2006). "Direct interaction of the molecular scaffolds POSH and JIP is required for apoptotic activation of JNKs." J Biol Chem **281**(22): 15517-24.

Kumar, S., J. Boehm and J. C. Lee (2003). "p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases." Nat Rev Drug Discov **2**(9): 717-26.

Kunz, A. and C. Iadecola (2009). "Cerebral vascular dysregulation in the ischemic brain." Handb Clin Neurol **92**: 283-305.

Kyriakis, J. M. and J. Avruch (1990). "pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine." J Biol Chem **265**(28): 17355-63.

Kyriakis, J. M., P. Banerjee, E. Nikolakaki, T. Dai, E. A. Rubie, M. F. Ahmad, J. Avruch and J. R. Woodgett (1994). "The stress-activated protein kinase subfamily of c-Jun kinases." Nature **369**(6476): 156-60.

Laabich, A., G. Li and N. G. Cooper (2001). "Characterization of apoptosis-genes associated with NMDA mediated cell death in the adult rat retina." Brain Res Mol Brain Res **91**(1-2): 34-42.

Labrie, V. and J. C. Roder (2010). "The involvement of the NMDA receptor D-serine/glycine site in the pathophysiology and treatment of schizophrenia." Neurosci Biobehav Rev **34**(3): 351-72.

Lai, T. W., W. C. Shyu and Y. T. Wang (2011). "Stroke intervention pathways: NMDA receptors and beyond." Trends Mol Med **17**(5): 266-75.

Lau, C. G. and R. S. Zukin (2007). "NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders." Nat Rev Neurosci **8**(6): 413-26.

Lau, D. and H. Bading (2009). "Synaptic activity-mediated suppression of p53 and induction of nuclear calcium-regulated neuroprotective genes promote survival through inhibition of mitochondrial permeability transition." J Neurosci **29**(14): 4420-9.

Lavezzari, G., J. McCallum, C. M. Dewey and K. W. Roche (2004). "Subunit-specific regulation of NMDA receptor endocytosis." J Neurosci **24**(28): 6383-91.

Lee, H. K. (2006). "Synaptic plasticity and phosphorylation." Pharmacol Ther **112**(3): 810-32.

Lee, J. K., J. H. Shin, S. G. Hwang, B. J. Gwag, A. C. McKee, J. Lee, N. W. Kowall, H. Ryu, D. S. Lim and E. J. Choi (2013). "MST1 functions as a key modulator of neurodegeneration in a mouse model of ALS." Proc Natl Acad Sci U S A.

Lee, T. H., Q. Huang, S. Oikemus, J. Shank, J. J. Ventura, N. Cusson, R. R. Vaillancourt, B. Su, R. J. Davis and M. A. Kelliher (2003). "The death domain

kinase RIP1 is essential for tumor necrosis factor alpha signaling to p38 mitogen-activated protein kinase." Mol Cell Biol **23**(22): 8377-85.

Lee, T. H., J. Shank, N. Cusson and M. A. Kelliher (2004). "The kinase activity of Rip1 is not required for tumor necrosis factor-alpha-induced IkappaB kinase or p38 MAP kinase activation or for the ubiquitination of Rip1 by Traf2." J Biol Chem **279**(32): 33185-91.

Lees, K. R. (1998). "Does neuroprotection improve stroke outcome?" Lancet **351**(9114): 1447-8.

Legos, J. J., B. McLaughlin, S. D. Skaper, P. J. Strijbos, A. A. Parsons, E. Aizenman, G. A. Herin, F. C. Barone and J. A. Erhardt (2002). "The selective p38 inhibitor SB-239063 protects primary neurons from mild to moderate excitotoxic injury." Eur J Pharmacol **447**(1): 37-42.

Lehtinen, M. K., Z. Yuan, P. R. Boag, Y. Yang, J. Villen, E. B. Becker, S. DiBacco, N. de la Iglesia, S. Gygi, T. K. Blackwell and A. Bonni (2006). "A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span." Cell **125**(5): 987-1001.

Lei, K. and R. J. Davis (2003). "JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis." Proc Natl Acad Sci U S A **100**(5): 2432-7.

Leveille, F., F. El Gaamouch, E. Gouix, M. Lecocq, D. Lobner, O. Nicole and A. Buisson (2008). "Neuronal viability is controlled by a functional relation between synaptic and extrasynaptic NMDA receptors." FASEB J **22**(12): 4258-71.

Leveille, F., S. Papadia, M. Fricker, K. F. Bell, F. X. Soriano, M. A. Martel, C. Puddifoot, M. Habel, D. J. Wyllie, C. Ikonomidou, A. M. Tolkovsky and G. E. Hardingham (2010). "Suppression of the intrinsic apoptosis pathway by synaptic activity." J Neurosci **30**(7): 2623-35.

Ley, R., K. E. Ewings, K. Hadfield, E. Howes, K. Balmanno and S. J. Cook (2004). "Extracellular signal-regulated kinases 1/2 are serum-stimulated "Bim(EL) kinases" that bind to the BH3-only protein Bim(EL) causing its phosphorylation and turnover." J Biol Chem **279**(10): 8837-47.

Li, B., N. Chen, T. Luo, Y. Otsu, T. H. Murphy and L. A. Raymond (2002). "Differential regulation of synaptic and extra-synaptic NMDA receptors." Nat Neurosci **5**(9): 833-4.

Li, H., M. Kobayashi, M. Blonska, Y. You and X. Lin (2006). "Ubiquitination of RIP is required for tumor necrosis factor alpha-induced NF-kappaB activation." J Biol Chem **281**(19): 13636-43.

Li, H., H. Zhu, C. J. Xu and J. Yuan (1998a). "Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis." Cell **94**(4): 491-501.

Li, J., T. McQuade, A. B. Siemer, J. Napetschnig, K. Moriwaki, Y. S. Hsiao, E. Damko, D. Moquin, T. Walz, A. McDermott, F. K. Chan and H. Wu (2012). "The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis." Cell **150**(2): 339-50.

Li, J. H., Y. H. Wang, B. B. Wolfe, K. E. Krueger, L. Corsi, G. Stocca and S. Vicini (1998b). "Developmental changes in localization of NMDA receptor subunits in primary cultures of cortical neurons." Eur J Neurosci **10**(5): 1704-15.

Li, L. L., V. Ginet, X. Liu, O. Vergun, M. Tuittila, M. Mathieu, C. Bonny, J. Puyal, A. C. Truttmann and M. J. Courtney (2013). "The nNOS-p38MAPK pathway is mediated by NOS1AP during neuronal death." J Neurosci **33**(19): 8185-201.

Li, L. Y., X. Luo and X. Wang (2001). "Endonuclease G is an apoptotic DNase when released from mitochondria." Nature **412**(6842): 95-9.

Li, Y., X. Yang, C. Ma, J. Qiao and C. Zhang (2008). "Necroptosis contributes to the NMDA-induced excitotoxicity in rat's cultured cortical neurons." Neurosci Lett **447**(2-3): 120-3.

Liang, G., C. D. Wolfgang, B. P. Chen, T. H. Chen and T. Hai (1996). "ATF3 gene. Genomic organization, promoter, and regulation." J Biol Chem **271**(3): 1695-701.

Lin, J. W., W. Ju, K. Foster, S. H. Lee, G. Ahmadian, M. Wyszynski, Y. T. Wang and M. Sheng (2000). "Distinct molecular mechanisms and divergent endocytotic pathways of AMPA receptor internalization." Nat Neurosci **3**(12): 1282-90.

Lipton, S. A. and P. A. Rosenberg (1994). "Excitatory amino acids as a final common pathway for neurologic disorders." N Engl J Med **330**(9): 613-22.

Lisman, J., H. Schulman and H. Cline (2002). "The molecular basis of CaMKII function in synaptic and behavioural memory." Nat Rev Neurosci **3**(3): 175-90.

Liu, C., M. Peng, A. M. Laties and R. Wen (1998). "Preconditioning with bright light evokes a protective response against light damage in the rat retina." J Neurosci **18**(4): 1337-44.

Liu, J. and A. Lin (2005). "Role of JNK activation in apoptosis: a double-edged sword." Cell Res **15**(1): 36-42.

Liu, R., L. Zhang, X. Lan, L. Li, T. T. Zhang, J. H. Sun and G. H. Du (2011). "Protection by borneol on cortical neurons against oxygen-glucose deprivation/reperfusion: involvement of anti-oxidation and anti-inflammation through nuclear transcription factor kappaappaB signaling pathway." Neuroscience **176**: 408-19.

Liu, S., L. Lau, J. Wei, D. Zhu, S. Zou, H. S. Sun, Y. Fu, F. Liu and Y. Lu (2004). "Expression of Ca(2+)-permeable AMPA receptor channels primes cell death in transient forebrain ischemia." Neuron **43**(1): 43-55.

Liu, Y., L. Tao, X. Fu, Y. Zhao and X. Xu (2013). "BDNF protects retinal neurons from hyperglycemia through the TrkB/ERK/MAPK pathway." Mol Med Rep **7**(6): 1773-8.

Liu, Y., T. P. Wong, M. Aarts, A. Rooyakkers, L. Liu, T. W. Lai, D. C. Wu, J. Lu, M. Tymianski, A. M. Craig and Y. T. Wang (2007). "NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo." J Neurosci **27**(11): 2846-57.

Liu, Y. F. (1998). "Expression of polyglutamine-expanded Huntingtin activates the SEK1-JNK pathway and induces apoptosis in a hippocampal neuronal cell line." J Biol Chem **273**(44): 28873-7.

Lo, E. H., T. Dalkara and M. A. Moskowitz (2003). "Mechanisms, challenges and opportunities in stroke." Nat Rev Neurosci **4**(5): 399-415.

Lonze, B. E., A. Riccio, S. Cohen and D. D. Ginty (2002). "Apoptosis, axonal growth defects, and degeneration of peripheral neurons in mice lacking CREB." Neuron **34**(3): 371-85.

Lu, C., F. Zhu, Y. Y. Cho, F. Tang, T. Zykova, W. Y. Ma, A. M. Bode and Z. Dong (2006). "Cell apoptosis: requirement of H2AX in DNA ladder formation, but not for the activation of caspase-3." Mol Cell **23**(1): 121-32.

Lu, Q., T. F. Rau, V. Harris, M. Johnson, D. J. Poulsen and S. M. Black (2011). "Increased p38 mitogen-activated protein kinase signaling is involved in the oxidative stress associated with oxygen and glucose deprivation in neonatal hippocampal slice cultures." Eur J Neurosci **34**(7): 1093-101.

Lu, Q., M. S. Wainwright, V. A. Harris, S. Aggarwal, Y. Hou, T. Rau, D. J. Poulsen and S. M. Black (2012). "Increased NADPH oxidase-derived superoxide is involved in the neuronal cell death induced by hypoxia-ischemia in neonatal hippocampal slice cultures." Free Radic Biol Med **53**(5): 1139-51.

Luciano, F., A. Jacquel, P. Colosetti, M. Herrant, S. Cagnol, G. Pages and P. Auberger (2003). "Phosphorylation of Bim-EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function." Oncogene **22**(43): 6785-93.

Luo, J., D. B. Kintner, G. E. Shull and D. Sun (2007). "ERK1/2-p90RSK-mediated phosphorylation of Na+/H+ exchanger isoform 1. A role in ischemic neuronal death." J Biol Chem **282**(38): 28274-84.

Ma, C., C. Ying, Z. Yuan, B. Song, D. Li, Y. Liu, B. Lai, W. Li, R. Chen, Y. P. Ching and M. Li (2007). "dp5/HRK is a c-Jun target gene and required for apoptosis induced by potassium deprivation in cerebellar granule neurons." J Biol Chem **282**(42): 30901-9.

MacDermott, A. B., M. L. Mayer, G. L. Westbrook, S. J. Smith and J. L. Barker (1986). "NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones." Nature **321**(6069): 519-22.

Makhatadze, N. J. (1998). "Tumor necrosis factor locus: genetic organisation and biological implications." Hum Immunol **59**(9): 571-9.

Maki, B. A., T. K. Aman, S. A. Amico-Ruvio, C. L. Kussius and G. K. Popescu (2012). "C-terminal domains of N-methyl-D-aspartic acid receptor modulate unitary channel conductance and gating." J Biol Chem **287**(43): 36071-80.

Malagelada, C., X. Xifro, A. Minano, J. Sabria and J. Rodriguez-Alvarez (2005). "Contribution of caspase-mediated apoptosis to the cell death caused by oxygen-glucose deprivation in cortical cell cultures." Neurobiol Dis **20**(1): 27-37.

Malenka, R. C. and R. A. Nicoll (1999). "Long-term potentiation--a decade of progress?" Science **285**(5435): 1870-4.

Manadas, B., A. R. Santos, K. Szabadfi, J. R. Gomes, S. D. Garbis, M. Fountoulakis and C. B. Duarte (2009). "BDNF-induced changes in the expression of the translation machinery in hippocampal neurons: protein levels and dendritic mRNA." J Proteome Res **8**(10): 4536-52.

Maroney, A. C., J. P. Finn, D. Bozyczko-Coyne, T. M. O'Kane, N. T. Neff, A. M. Tolkovsky, D. S. Park, C. Y. Yan, C. M. Troy and L. A. Greene (1999). "CEP-1347 (KT7515), an inhibitor of JNK activation, rescues sympathetic neurons and neuronally differentiated PC12 cells from death evoked by three distinct insults." J Neurochem **73**(5): 1901-12.

Martel, M. A., T. J. Ryan, K. F. Bell, J. H. Fowler, A. McMahon, B. Al-Mubarak, N. H. Komiyama, K. Horsburgh, P. C. Kind, S. G. Grant, D. J. Wyllie and G. E. Hardingham (2012). "The subtype of GluN2 C-terminal domain determines the response to excitotoxic insults." Neuron **74**(3): 543-56.

Martel, M. A., F. X. Soriano, P. Baxter, C. Rickman, R. Duncan, D. J. Wyllie and G. E. Hardingham (2009a). "Inhibiting pro-death NMDA receptor signaling dependent on the NR2 PDZ ligand may not affect synaptic function or synaptic NMDA receptor signaling to gene expression." Channels (Austin) **3**(1): 12-5.

Martel, M. A., D. J. Wyllie and G. E. Hardingham (2009b). "In developing hippocampal neurons, NR2B-containing N-methyl-D-aspartate receptors (NMDARs) can

mediate signaling to neuronal survival and synaptic potentiation, as well as neuronal death." Neuroscience **158**(1): 334-43.

Martin-Villalba, A., M. Hahne, S. Kleber, J. Vogel, W. Falk, J. Schenkel and P. H. Krammer (2001). "Therapeutic neutralization of CD95-ligand and TNF attenuates brain damage in stroke." Cell Death Differ **8**(7): 679-86.

Martin, J. H., A. A. Mohit and C. A. Miller (1996). "Developmental expression in the mouse nervous system of the p493F12 SAP kinase." Brain Res Mol Brain Res **35**(1-2): 47-57.

Martin, R. L., H. G. Lloyd and A. I. Cowan (1994). "The early events of oxygen and glucose deprivation: setting the scene for neuronal death?" Trends Neurosci **17**(6): 251-7.

Martinez-Sanchez, M., F. Striggow, U. H. Schroder, S. Kahlert, K. G. Reymann and G. Reiser (2004). "Na(+) and Ca(2+) homeostasis pathways, cell death and protection after oxygen-glucose-deprivation in organotypic hippocampal slice cultures." Neuroscience **128**(4): 729-40.

Matthews, R. P., C. R. Guthrie, L. M. Wailes, X. Zhao, A. R. Means and G. S. McKnight (1994). "Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression." Mol Cell Biol **14**(9): 6107-16.

Maundrell, K., B. Antonsson, E. Magnenat, M. Camps, M. Muda, C. Chabert, C. Gillieron, U. Boschert, E. Vial-Knecht, J. C. Martinou and S. Arkinstall (1997). "Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1." J Biol Chem **272**(40): 25238-42.

Mayer, M. L. and G. L. Westbrook (1987). "Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurones." J Physiol **394**: 501-27.

Mayer, M. L., G. L. Westbrook and P. B. Guthrie (1984). "Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones." Nature **309**(5965): 261-3.

Mayr, B. and M. Montminy (2001). "Transcriptional regulation by the phosphorylation-dependent factor CREB." Nat Rev Mol Cell Biol **2**(8): 599-609.

McDonald, P. H., C. W. Chow, W. E. Miller, S. A. Laporte, M. E. Field, F. T. Lin, R. J. Davis and R. J. Lefkowitz (2000). "Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3." Science **290**(5496): 1574-7.

Meade, A. J., B. P. Meloni, J. Cross, A. J. Bakker, M. W. Fear, F. L. Mastaglia, P. M. Watt and N. W. Knuckey (2010). "AP-1 inhibitory peptides are neuroprotective

following acute glutamate excitotoxicity in primary cortical neuronal cultures." J Neurochem **112**(1): 258-70.

Mehta, A., M. Prabhakar, P. Kumar, R. Deshmukh and P. L. Sharma (2013). "Excitotoxicity: bridge to various triggers in neurodegenerative disorders." Eur J Pharmacol **698**(1-3): 6-18.

Mei, Y., Z. Yuan, B. Song, D. Li, C. Ma, C. Hu, Y. P. Ching and M. Li (2008). "Activating transcription factor 3 up-regulated by c-Jun NH(2)-terminal kinase/c-Jun contributes to apoptosis induced by potassium deprivation in cerebellar granule neurons." Neuroscience **151**(3): 771-9.

Meloni, B. P., A. J. Meade, D. Kitikomolsuk and N. W. Knuckey (2011). "Characterisation of neuronal cell death in acute and delayed in vitro ischemia (oxygen-glucose deprivation) models." J Neurosci Methods **195**(1): 67-74.

Micheau, O., M. Thome, P. Schneider, N. Holler, J. Tschopp, D. W. Nicholson, C. Briand and M. G. Grutter (2002). "The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex." J Biol Chem **277**(47): 45162-71.

Micheau, O. and J. Tschopp (2003). "Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes." Cell **114**(2): 181-90.

Minden, A., A. Lin, T. Smeal, B. Derijard, M. Cobb, R. Davis and M. Karin (1994). "c-Jun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK subgroup of mitogen-activated protein kinases." Mol Cell Biol **14**(10): 6683-8.

Miyawaki, T., T. Mashiko, D. Ofengeim, R. J. Flannery, K. M. Noh, S. Fujisawa, L. Bonanni, M. V. Bennett, R. S. Zukin and E. A. Jonas (2008). "Ischemic preconditioning blocks BAD translocation, Bcl-xL cleavage, and large channel activity in mitochondria of postischemic hippocampal neurons." Proc Natl Acad Sci U S A **105**(12): 4892-7.

Mohit, A. A., J. H. Martin and C. A. Miller (1995). "p493F12 kinase: a novel MAP kinase expressed in a subset of neurons in the human nervous system." Neuron **14**(1): 67-78.

Montori, S., S. Dos-Anjos, B. Martinez-Villayandre, M. M. Regueiro-Purrinos, J. M. Gonzalo-Orden, D. Ruano and A. Fernandez-Lopez (2010). "Age and meloxicam attenuate the ischemia/reperfusion-induced down-regulation in the NMDA receptor genes." Neurochem Int **56**(8): 878-85.

Monyer, H., N. Burnashev, D. J. Laurie, B. Sakmann and P. H. Seeburg (1994). "Developmental and regional expression in the rat brain and functional properties of four NMDA receptors." Neuron **12**(3): 529-40.

Morrison, D. K. and R. J. Davis (2003). "Regulation of MAP kinase signaling modules by scaffold proteins in mammals." Annu Rev Cell Dev Biol **19**: 91-118.

Moskowitz, M. A., E. H. Lo and C. Iadecola (2010). "The science of stroke: mechanisms in search of treatments." Neuron **67**(2): 181-98.

Moujalled, D. M., W. D. Cook, T. Okamoto, J. Murphy, K. E. Lawlor, J. E. Vince and D. L. Vaux (2013). "TNF can activate RIPK3 and cause programmed necrosis in the absence of RIPK1." Cell Death Dis **4**: e465.

Mu, Y., T. Otsuka, A. C. Horton, D. B. Scott and M. D. Ehlers (2003). "Activity-dependent mRNA splicing controls ER export and synaptic delivery of NMDA receptors." Neuron **40**(3): 581-94.

Murphy, L. O. and J. Blenis (2006). "MAPK signal specificity: the right place at the right time." Trends Biochem Sci **31**(5): 268-75.

Narayan, N., I. H. Lee, R. Borenstein, J. Sun, R. Wong, G. Tong, M. M. Fergusson, J. Liu, Rovira, II, H. L. Cheng, G. Wang, M. Gucek, D. Lombard, F. W. Alt, M. N. Sack, E. Murphy, L. Cao and T. Finkel (2012). "The NAD-dependent deacetylase SIRT2 is required for programmed necrosis." Nature **492**(7428): 199-204.

Nath, R., A. Probert, Jr., K. M. McGinnis and K. K. Wang (1998). "Evidence for activation of caspase-3-like protease in excitotoxin- and hypoxia/hypoglycemia-injured neurons." J Neurochem **71**(1): 186-95.

Neubert, M., D. A. Ridder, P. Bargiotas, S. Akira and M. Schwaninger (2011). "Acute inhibition of TAK1 protects against neuronal death in cerebral ischemia." Cell Death Differ **18**(9): 1521-30.

Newcomb-Fernandez, J. K., X. Zhao, B. R. Pike, K. K. Wang, A. Kampfl, R. Beer, S. M. DeFord and R. L. Hayes (2001). "Concurrent assessment of calpain and caspase-3 activation after oxygen-glucose deprivation in primary septo-hippocampal cultures." J Cereb Blood Flow Metab **21**(11): 1281-94.

Neyton, J. and P. Paoletti (2006). "Relating NMDA receptor function to receptor subunit composition: limitations of the pharmacological approach." J Neurosci **26**(5): 1331-3.

Nong, Y., Y. Q. Huang, W. Ju, L. V. Kalia, G. Ahmadian, Y. T. Wang and M. W. Salter (2003). "Glycine binding primes NMDA receptor internalization." Nature **422**(6929): 302-7.

Northington, F. J., R. Chavez-Valdez, E. M. Graham, S. Razdan, E. B. Gauda and L. J. Martin (2011). "Necrostatin decreases oxidative damage, inflammation, and injury after neonatal HI." J Cereb Blood Flow Metab **31**(1): 178-89.

Nowak, L., P. Bregestovski, P. Ascher, A. Herbet and A. Prochiantz (1984). "Magnesium gates glutamate-activated channels in mouse central neurones." Nature **307**(5950): 462-5.

O'Donnell, M. A., E. Perez-Jimenez, A. Oberst, A. Ng, R. Massoumi, R. Xavier, D. R. Green and A. T. Ting (2011). "Caspase 8 inhibits programmed necrosis by processing CYLD." Nat Cell Biol **13**(12): 1437-42.

O'Leary, H., W. H. Liu, J. M. Rorabaugh, S. J. Coultrap and K. U. Bayer (2011). "Nucleotides and phosphorylation bi-directionally modulate Ca2+/calmodulin-dependent protein kinase II (CaMKII) binding to the N-methyl-D-aspartate (NMDA) receptor subunit GluN2B." J Biol Chem **286**(36): 31272-81.

Oberst, A., C. P. Dillon, R. Weinlich, L. L. McCormick, P. Fitzgerald, C. Pop, R. Hakem, G. S. Salvesen and D. R. Green (2011). "Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis." Nature **471**(7338): 363-7.

Oerlemans, M. I., J. Liu, F. Arslan, K. den Ouden, B. J. van Middelaar, P. A. Doevendans and J. P. Sluijter (2012). "Inhibition of RIP1-dependent necrosis prevents adverse cardiac remodeling after myocardial ischemia-reperfusion in vivo." Basic Res Cardiol **107**(4): 270.

Ogden, K. K. and S. F. Traynelis (2011). "New advances in NMDA receptor pharmacology." Trends Pharmacol Sci **32**(12): 726-33.

Okuno, S., A. Saito, T. Hayashi and P. H. Chan (2004). "The c-Jun N-terminal protein kinase signaling pathway mediates Bax activation and subsequent neuronal apoptosis through interaction with Bim after transient focal cerebral ischemia." J Neurosci **24**(36): 7879-87.

Olney, J. W. (1969). "Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate." Science **164**(3880): 719-21.

Owens, D. M. and S. M. Keyse (2007). "Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases." Oncogene **26**(22): 3203-13.

Ozawa, H., S. Shioda, K. Dohi, H. Matsumoto, H. Mizushima, C. J. Zhou, H. Funahashi, Y. Nakai, S. Nakajo and K. Matsumoto (1999). "Delayed neuronal cell death in the rat hippocampus is mediated by the mitogen-activated protein kinase signal transduction pathway." Neurosci Lett **262**(1): 57-60.

Papadia, S., F. X. Soriano, F. Leveille, M. A. Martel, K. A. Dakin, H. H. Hansen, A. Kaindl, M. Sifringer, J. Fowler, V. Stefovska, G. McKenzie, M. Craigon, R. Corriveau, P. Ghazal, K. Horsburgh, B. A. Yankner, D. J. Wyllie, C. Ikonomidou and G. E. Hardingham (2008). "Synaptic NMDA receptor activity boosts intrinsic antioxidant defenses." Nat Neurosci **11**(4): 476-87.

Papadia, S., P. Stevenson, N. R. Hardingham, H. Bading and G. E. Hardingham (2005). "Nuclear Ca2+ and the cAMP response element-binding protein family mediate a late phase of activity-dependent neuroprotection." J Neurosci **25**(17): 4279-87.

Papouin, T., L. Ladepeche, J. Ruel, S. Sacchi, M. Labasque, M. Hanini, L. Groc, L. Pollegioni, J. P. Mothet and S. H. Oliet (2012). "Synaptic and extrasynaptic NMDA receptors are gated by different endogenous coagonists." Cell **150**(3): 633-46.

Parker, C. G., J. Hunt, K. Diener, M. McGinley, B. Soriano, G. A. Keesler, J. Bray, Z. Yao, X. S. Wang, T. Kohno and H. S. Lichenstein (1998). "Identification of stathmin as a novel substrate for p38 delta." Biochem Biophys Res Commun **249**(3): 791-6.

Pei, D. S., X. T. Wang, Y. Liu, Y. F. Sun, Q. H. Guan, W. Wang, J. Z. Yan, Y. Y. Zong, T. L. Xu and G. Y. Zhang (2006). "Neuroprotection against ischaemic brain injury by a GluR6-9c peptide containing the TAT protein transduction sequence." Brain **129**(Pt 2): 465-79.

Pellegrini-Giampietro, D. E., J. A. Gorter, M. V. Bennett and R. S. Zukin (1997). "The GluR2 (GluR-B) hypothesis: Ca(2+)-permeable AMPA receptors in neurological disorders." Trends Neurosci **20**(10): 464-70.

Petito, C. K., E. Feldmann, W. A. Pulsinelli and F. Plum (1987). "Delayed hippocampal damage in humans following cardiorespiratory arrest." Neurology **37**(8): 1281-6.

Petralia, R. S., Y. X. Wang, F. Hua, Z. Yi, A. Zhou, L. Ge, F. A. Stephenson and R. J. Wenthold (2010). "Organization of NMDA receptors at extrasynaptic locations." Neuroscience **167**(1): 68-87.

Pierce, K. L. and R. J. Lefkowitz (2001). "Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors." Nat Rev Neurosci **2**(10): 727-33.

Plesnila, N., S. Zinkel, D. A. Le, S. Amin-Hanjani, Y. Wu, J. Qiu, A. Chiarugi, S. S. Thomas, D. S. Kohane, S. J. Korsmeyer and M. A. Moskowitz (2001). "BID mediates neuronal cell death after oxygen/ glucose deprivation and focal cerebral ischemia." Proc Natl Acad Sci U S A **98**(26): 15318-23.

Popoli, M., Z. Yan, B. S. McEwen and G. Sanacora (2012). "The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission." Nat Rev Neurosci **13**(1): 22-37.

Prybylowski, K., K. Chang, N. Sans, L. Kan, S. Vicini and R. J. Wenthold (2005). "The synaptic localization of NR2B-containing NMDA receptors is controlled by interactions with PDZ proteins and AP-2." Neuron **47**(6): 845-57.

Pulverer, B. J., J. M. Kyriakis, J. Avruch, E. Nikolakaki and J. R. Woodgett (1991). "Phosphorylation of c-jun mediated by MAP kinases." Nature **353**(6345): 670-4.

Punnakkal, P., P. Jendritza and G. Kohr (2012). "Influence of the intracellular GluN2 C-terminal domain on NMDA receptor function." Neuropharmacology **62**(5-6): 1985-92.

Putcha, G. V., K. L. Moulder, J. P. Golden, P. Bouillet, J. A. Adams, A. Strasser and E. M. Johnson (2001). "Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis." Neuron **29**(3): 615-28.

Qi, M. and E. A. Elion (2005). "MAP kinase pathways." J Cell Sci **118**(Pt 16): 3569-72.

Qin, J., J. Yao, G. Cui, H. Xiao, T. W. Kim, J. Fraczek, P. Wightman, S. Sato, S. Akira, A. Puel, J. L. Casanova, B. Su and X. Li (2006). "TLR8-mediated NF-kappaB and JNK activation are TAK1-independent and MEKK3-dependent." J Biol Chem **281**(30): 21013-21.

Raman, M., W. Chen and M. H. Cobb (2007). "Differential regulation and properties of MAPKs." Oncogene **26**(22): 3100-12.

Rao, J. S., M. Kellom, H. W. Kim, S. I. Rapoport and E. A. Reese (2012). "Neuroinflammation and synaptic loss." Neurochem Res **37**(5): 903-10.

Raveendran, R., S. Devi Suma Priya, M. Mayadevi, M. Steephan, T. R. Santhoshkumar, J. Cheriyan, R. Sanalkumar, K. K. Pradeep, J. James and R. V. Omkumar (2009). "Phosphorylation status of the NR2B subunit of NMDA receptor regulates its interaction with calcium/calmodulin-dependent protein kinase II." J Neurochem **110**(1): 92-105.

Rebsamen, M., L. X. Heinz, E. Meylan, M. C. Michallet, K. Schroder, K. Hofmann, J. Vazquez, C. A. Benedict and J. Tschopp (2009). "DAI/ZBP1 recruits RIP1 and RIP3 through RIP homotypic interaction motifs to activate NF-kappaB." EMBO Rep **10**(8): 916-22.

Repici, M., C. Centeno, S. Tomasi, G. Forloni, C. Bonny, A. Vercelli and T. Borsello (2007). "Time-course of c-Jun N-terminal kinase activation after cerebral ischemia and effect of D-JNKI1 on c-Jun and caspase-3 activation." Neuroscience **150**(1): 40-9.

Rieckmann, P., M. Albrecht, B. Kitze, T. Weber, H. Tumani, A. Broocks, W. Luer, A. Helwig and S. Poser (1995). "Tumor necrosis factor-alpha messenger RNA expression in patients with relapsing-remitting multiple sclerosis is associated with disease activity." Ann Neurol **37**(1): 82-8.

Roche, K. W., S. Standley, J. McCallum, C. Dune Ly, M. D. Ehlers and R. J. Wenthold (2001). "Molecular determinants of NMDA receptor internalization." Nat Neurosci **4**(8): 794-802.

Rock, D. M. and R. L. Macdonald (1995). "Polyamine regulation of N-methyl-D-aspartate receptor channels." Annu Rev Pharmacol Toxicol **35**: 463-82.

Romera, C., O. Hurtado, S. H. Botella, I. Lizasoain, A. Cardenas, P. Fernandez-Tome, J. C. Leza, P. Lorenzo and M. A. Moro (2004). "In vitro ischemic tolerance involves upregulation of glutamate transport partly mediated by the TACE/ADAM17-tumor necrosis factor-alpha pathway." J Neurosci **24**(6): 1350-7.

Rosenbaum, D. M., A. Degterev, J. David, P. S. Rosenbaum, S. Roth, J. C. Grotta, G. D. Cuny, J. Yuan and S. I. Savitz (2010). "Necroptosis, a novel form of caspase-independent cell death, contributes to neuronal damage in a retinal ischemia-reperfusion injury model." J Neurosci Res **88**(7): 1569-76.

Rubio-Perez, J. M. and J. M. Morillas-Ruiz (2012). "A review: inflammatory process in Alzheimer's disease, role of cytokines." ScientificWorldJournal **2012**: 756357.

Ryan, T. J., R. D. Emes, S. G. Grant and N. H. Komiyama (2008). "Evolution of NMDA receptor cytoplasmic interaction domains: implications for organisation of synaptic signalling complexes." BMC Neurosci **9**: 6.

Saelens, X., N. Festjens, L. Vande Walle, M. van Gurp, G. van Loo and P. Vandenabeele (2004). "Toxic proteins released from mitochondria in cell death." Oncogene **23**(16): 2861-74.

Sakahira, H., M. Enari and S. Nagata (1998). "Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis." Nature **391**(6662): 96-9.

Salih, D. A. and A. Brunet (2008). "FoxO transcription factors in the maintenance of cellular homeostasis during aging." Curr Opin Cell Biol **20**(2): 126-36.

Salmeron, A., T. B. Ahmad, G. W. Carlile, D. Pappin, R. P. Narsimhan and S. C. Ley (1996). "Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase." EMBO J **15**(4): 817-26.

Salojin, K. V., I. B. Owusu, K. A. Millerchip, M. Potter, K. A. Platt and T. Oravecz (2006). "Essential role of MAPK phosphatase-1 in the negative control of innate immune responses." J Immunol **176**(3): 1899-907.

Sans, N., P. Y. Wang, Q. Du, R. S. Petralia, Y. X. Wang, S. Nakka, J. B. Blumer, I. G. Macara and R. J. Wenthold (2005). "mPins modulates PSD-95 and SAP102 trafficking and influences NMDA receptor surface expression." Nat Cell Biol **7**(12): 1179-90.

Santos, A. E., C. B. Duarte, M. Iizuka, E. L. Barsoumian, J. Ham, M. C. Lopes, A. P. Carvalho and A. L. Carvalho (2006). "Excitotoxicity mediated by Ca2+-permeable GluR4-containing AMPA receptors involves the AP-1 transcription factor." Cell Death Differ **13**(4): 652-60.

Santos, A. R. and C. B. Duarte (2008). "Validation of internal control genes for expression studies: effects of the neurotrophin BDNF on hippocampal neurons." J Neurosci Res **86**(16): 3684-92.

Saporito, M. S., B. A. Thomas and R. W. Scott (2000). "MPTP activates c-Jun NH(2)-terminal kinase (JNK) and its upstream regulatory kinase MKK4 in nigrostriatal neurons in vivo." J Neurochem **75**(3): 1200-8.

Sato, S., H. Sanjo, K. Takeda, J. Ninomiya-Tsuji, M. Yamamoto, T. Kawai, K. Matsumoto, O. Takeuchi and S. Akira (2005). "Essential function for the kinase TAK1 in innate and adaptive immune responses." Nat Immunol **6**(11): 1087-95.

Sattler, R. and M. Tymianski (2001). "Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death." Mol Neurobiol **24**(1-3): 107-29.

Sattler, R., Z. Xiong, W. Y. Lu, M. Hafner, J. F. MacDonald and M. Tymianski (1999). "Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein." Science **284**(5421): 1845-8.

Savage, M. J., Y. G. Lin, J. R. Ciallella, D. G. Flood and R. W. Scott (2002). "Activation of c-Jun N-terminal kinase and p38 in an Alzheimer's disease model is associated with amyloid deposition." J Neurosci **22**(9): 3376-85.

Scaffidi, C., I. Schmitz, P. H. Krammer and M. E. Peter (1999). "The role of c-FLIP in modulation of CD95-induced apoptosis." J Biol Chem **274**(3): 1541-8.

Scheller, C., S. Sopper, C. Ehrhardt, E. Flory, P. Chen, E. Koutsilieri, S. Ludwig, V. ter Meulen and C. Jassoy (2002). "Caspase inhibitors induce a switch from apoptotic to proinflammatory signaling in CD95-stimulated T lymphocytes." Eur J Immunol **32**(9): 2471-80.

Scimemi, A., A. Fine, D. M. Kullmann and D. A. Rusakov (2004). "NR2B-containing receptors mediate cross talk among hippocampal synapses." J Neurosci **24**(20): 4767-77.

Scott, D. B., I. Michailidis, Y. Mu, D. Logothetis and M. D. Ehlers (2004). "Endocytosis and degradative sorting of NMDA receptors by conserved membrane-proximal signals." J Neurosci **24**(32): 7096-109.

Segura Torres, J. E., V. Chaparro-Huerta, M. C. Rivera Cervantres, R. Montes-Gonzalez, M. E. Flores Soto and C. Beas-Zarate (2006). "Neuronal cell death due to glutamate excitotocity is mediated by p38 activation in the rat cerebral cortex." Neurosci Lett **403**(3): 233-8.

Semenova, M. M., A. M. Maki-Hokkonen, J. Cao, V. Komarovski, K. M. Forsberg, M. Koistinaho, E. T. Coffey and M. J. Courtney (2007). "Rho mediates calcium-dependent activation of p38alpha and subsequent excitotoxic cell death." Nat Neurosci **10**(4): 436-43.

Shackelford, D. A. and R. Y. Yeh (2006). "Modulation of ERK and JNK activity by transient forebrain ischemia in rats." J Neurosci Res **83**(3): 476-88.

Shen, H. M., Y. Lin, S. Choksi, J. Tran, T. Jin, L. Chang, M. Karin, J. Zhang and Z. G. Liu (2004). "Essential roles of receptor-interacting protein and TRAF2 in oxidative stress-induced cell death." Mol Cell Biol **24**(13): 5914-22.

Shen, K. and T. Meyer (1999). "Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation." Science **284**(5411): 162-6.

Sheng, M., J. Cummings, L. A. Roldan, Y. N. Jan and L. Y. Jan (1994). "Changing subunit composition of heteromeric NMDA receptors during development of rat cortex." Nature **368**(6467): 144-7.

Shim, J. H., C. Xiao, A. E. Paschal, S. T. Bailey, P. Rao, M. S. Hayden, K. Y. Lee, C. Bussey, M. Steckel, N. Tanaka, G. Yamada, S. Akira, K. Matsumoto and S. Ghosh (2005). "TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo." Genes Dev **19**(22): 2668-81.

Shinozaki, Y., Y. Sato, S. Koizumi, Y. Ohno, T. Nagao and K. Inoue (2007). "Retinoic acids acting through retinoid receptors protect hippocampal neurons from oxygen-glucose deprivation-mediated cell death by inhibition of c-jun-N-terminal kinase and p38 mitogen-activated protein kinase." Neuroscience **147**(1): 153-63.

Shirakabe, K., K. Yamaguchi, H. Shibuya, K. Irie, S. Matsuda, T. Moriguchi, Y. Gotoh, K. Matsumoto and E. Nishida (1997). "TAK1 mediates the ceramide signaling to stress-activated protein kinase/c-Jun N-terminal kinase." J Biol Chem **272**(13): 8141-4.

Silva, R. M., C. Y. Kuan, P. Rakic and R. E. Burke (2005). "Mixed lineage kinase-c-jun N-terminal kinase signaling pathway: a new therapeutic target in Parkinson's disease." Mov Disord **20**(6): 653-64.

Siman, R., J. C. Noszek and C. Kegerise (1989). "Calpain I activation is specifically related to excitatory amino acid induction of hippocampal damage." J Neurosci **9**(5): 1579-90.

Simenc, J. and M. Lipnik-Stangelj (2012). "Staurosporine induces different cell death forms in cultured rat astrocytes." Radiol Oncol **46**(4): 312-20.

Slee, E. A., C. Adrain and S. J. Martin (2001). "Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis." J Biol Chem **276**(10): 7320-6.

Sluss, H. K. and R. J. Davis (2006). "H2AX is a target of the JNK signaling pathway that is required for apoptotic DNA fragmentation." Mol Cell **23**(2): 152-3.

Snyder, E. M., B. D. Philpot, K. M. Huber, X. Dong, J. R. Fallon and M. F. Bear (2001). "Internalization of ionotropic glutamate receptors in response to mGluR activation." Nat Neurosci **4**(11): 1079-85.

Sobolevsky, A. I., M. P. Rosconi and E. Gouaux (2009). "X-ray structure, symmetry and mechanism of an AMPA-subtype glutamate receptor." Nature **462**(7274): 745-56.

Song, B., B. Xie, C. Wang and M. Li (2011). "Caspase-3 is a target gene of c-Jun:ATF2 heterodimers during apoptosis induced by activity deprivation in cerebellar granule neurons." Neurosci Lett **505**(2): 76-81.

Songyang, Z., K. P. Lu, Y. T. Kwon, L. H. Tsai, O. Filhol, C. Cochet, D. A. Brickey, T. R. Soderling, C. Bartleson, D. J. Graves, A. J. DeMaggio, M. F. Hoekstra, J. Blenis, T. Hunter and L. C. Cantley (1996). "A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, CDK5, and Erk1." Mol Cell Biol **16**(11): 6486-93.

Soriano, F. X., M. A. Martel, S. Papadia, A. Vaslin, P. Baxter, C. Rickman, J. Forder, M. Tymianski, R. Duncan, M. Aarts, P. Clarke, D. J. Wyllie and G. E. Hardingham (2008). "Specific targeting of pro-death NMDA receptor signals with differing reliance on the NR2B PDZ ligand." J Neurosci **28**(42): 10696-710.

Stanika, R. I., N. B. Pivovarova, C. A. Brantner, C. A. Watts, C. A. Winters and S. B. Andrews (2009). "Coupling diverse routes of calcium entry to mitochondrial dysfunction and glutamate excitotoxicity." Proc Natl Acad Sci U S A **106**(24): 9854-9.

Strack, S. and R. J. Colbran (1998). "Autophosphorylation-dependent targeting of calcium/ calmodulin-dependent protein kinase II by the NR2B subunit of the N-methyl- D-aspartate receptor." J Biol Chem **273**(33): 20689-92.

Strassburger, M., H. Braun and K. G. Reymann (2008). "Anti-inflammatory treatment with the p38 mitogen-activated protein kinase inhibitor SB239063 is neuroprotective, decreases the number of activated microglia and facilitates neurogenesis in oxygen-glucose-deprived hippocampal slice cultures." Eur J Pharmacol **592**(1-3): 55-61.

Sugino, T., K. Nozaki, Y. Takagi, I. Hattori, N. Hashimoto, T. Moriguchi and E. Nishida (2000). "Activation of mitogen-activated protein kinases after transient forebrain ischemia in gerbil hippocampus." J Neurosci **20**(12): 4506-14.

Sun, L., H. Wang, Z. Wang, S. He, S. Chen, D. Liao, L. Wang, J. Yan, W. Liu, X. Lei and X. Wang (2012). "Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase." Cell **148**(1-2): 213-27.

Sun, T., N. Yu, L. K. Zhai, N. Li, C. Zhang, L. Zhou, Z. Huang, X. Y. Jiang, Y. Shen and Z. Y. Chen (2013). "c-Jun NH2-terminal kinase (JNK)-interacting protein-3 (JIP3) regulates neuronal axon elongation in a kinesin- and JNK-dependent manner." J Biol Chem **288**(20): 14531-43.

Sun, X., J. Yin, M. A. Starovasnik, W. J. Fairbrother and V. M. Dixit (2002). "Identification of a novel homotypic interaction motif required for the phosphorylation of receptor-interacting protein (RIP) by RIP3." J Biol Chem **277**(11): 9505-11.

Szydlowska, K. and M. Tymianski (2010). "Calcium, ischemia and excitotoxicity." Cell Calcium **47**(2): 122-9.

Takeda, K., R. Shimozono, T. Noguchi, T. Umeda, Y. Morimoto, I. Naguro, K. Tobiume, M. Saitoh, A. Matsuzawa and H. Ichijo (2007). "Apoptosis signal-regulating kinase (ASK) 2 functions as a mitogen-activated protein kinase kinase kinase in a heteromeric complex with ASK1." J Biol Chem **282**(10): 7522-31.

Tararuk, T., N. Ostman, W. Li, B. Bjorkblom, A. Padzik, J. Zdrojewska, V. Hongisto, T. Herdegen, W. Konopka, M. J. Courtney and E. T. Coffey (2006). "JNK1 phosphorylation of SCG10 determines microtubule dynamics and axodendritic length." J Cell Biol **173**(2): 265-77.

Taylor, D. M., R. Moser, E. Regulier, L. Breuillaud, M. Dixon, A. A. Beesen, L. Elliston, F. Silva Santos Mde, J. Kim, L. Jones, D. R. Goldstein, R. J. Ferrante and R. Luthi-Carter (2013). "MAP kinase phosphatase 1 (MKP-1/DUSP1) is neuroprotective in Huntington's disease via additive effects of JNK and p38 inhibition." J Neurosci **33**(6): 2313-25.

Taylor, R. C., S. P. Cullen and S. J. Martin (2008). "Apoptosis: controlled demolition at the cellular level." Nat Rev Mol Cell Biol **9**(3): 231-41.

Tenev, T., K. Bianchi, M. Darding, M. Broemer, C. Langlais, F. Wallberg, A. Zachariou, J. Lopez, M. MacFarlane, K. Cain and P. Meier (2011). "The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs." Mol Cell **43**(3): 432-48.

Terasaki, Y., T. Sasaki, Y. Yagita, S. Okazaki, Y. Sugiyama, N. Oyama, E. Omura-Matsuoka, S. Sakoda and K. Kitagawa (2010). "Activation of NR2A receptors induces ischemic tolerance through CREB signaling." J Cereb Blood Flow Metab **30**(8): 1441-9.

Tezuka, Y., M. Okada, Y. Tada, J. Yamauchi, H. Nishigori and A. Sanbe (2013). "Regulation of neurite growth by inorganic pyrophosphatase 1 via JNK dephosphorylation." PLoS One **8**(4): e61649.

Thevenet, J., A. Angelillo-Scherrer, M. Price and L. Hirt (2009). "Coagulation factor Xa activates thrombin in ischemic neural tissue." J Neurochem **111**(3): 828-36.

Thoenen, H., Y. A. Barde, A. M. Davies and J. E. Johnson (1987). "Neurotrophic factors and neuronal death." Ciba Found Symp **126**: 82-95.

Thomas, C. G., A. J. Miller and G. L. Westbrook (2006). "Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons." J Neurophysiol **95**(3): 1727-34.

Thomas, G. M., D. T. Lin, M. Nuriya and R. L. Huganir (2008a). "Rapid and bi-directional regulation of AMPA receptor phosphorylation and trafficking by JNK." EMBO J **27**(2): 361-72.

Thomas, T., M. Timmer, K. Cesnulevicius, E. Hitti, A. Kotlyarov and M. Gaestel (2008b). "MAPKAP kinase 2-deficiency prevents neurons from cell death by reducing neuroinflammation--relevance in a mouse model of Parkinson's disease." J Neurochem **105**(5): 2039-52.

Tia, S., J. F. Wang, N. Kotchabhakdi and S. Vicini (1996). "Distinct deactivation and desensitization kinetics of recombinant GABAA receptors." Neuropharmacology **35**(9-10): 1375-82.

Tian, H., Q. Zhang, H. Li and G. Zhang (2003). "Antioxidant N-acetylcysteine and AMPA/KA receptor antagonist DNQX inhibited mixed lineage kinase-3 activation following cerebral ischemia in rat hippocampus." Neurosci Res **47**(1): 47-53.

Ting, A. T., F. X. Pimentel-Muinos and B. Seed (1996). "RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis." EMBO J **15**(22): 6189-96.

Tobiume, K., A. Matsuzawa, T. Takahashi, H. Nishitoh, K. Morita, K. Takeda, O. Minowa, K. Miyazono, T. Noda and H. Ichijo (2001). "ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis." EMBO Rep **2**(3): 222-8.

Tokunaga, F., H. Nishimasu, R. Ishitani, E. Goto, T. Noguchi, K. Mio, K. Kamei, A. Ma, K. Iwai and O. Nureki (2012). "Specific recognition of linear polyubiquitin by A20 zinc finger 7 is involved in NF-kappaB regulation." EMBO J **31**(19): 3856-70.

Tokunaga, F., S. Sakata, Y. Saeki, Y. Satomi, T. Kirisako, K. Kamei, T. Nakagawa, M. Kato, S. Murata, S. Yamaoka, M. Yamamoto, S. Akira, T. Takao, K. Tanaka and K. Iwai (2009). "Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation." Nat Cell Biol **11**(2): 123-32.

Tournier, C., C. Dong, T. K. Turner, S. N. Jones, R. A. Flavell and R. J. Davis (2001). "MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines." Genes Dev **15**(11): 1419-26.

Tournier, C., P. Hess, D. D. Yang, J. Xu, T. K. Turner, A. Nimnual, D. Bar-Sagi, S. N. Jones, R. A. Flavell and R. J. Davis (2000). "Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway." Science **288**(5467): 870-4.

Tovar, K. R. and G. L. Westbrook (1999). "The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro." J Neurosci **19**(10): 4180-8.

Tovar, K. R. and G. L. Westbrook (2002). "Mobile NMDA receptors at hippocampal synapses." Neuron **34**(2): 255-64.

Towers, E., J. Gilley, R. Randall, R. Hughes, M. Kristiansen and J. Ham (2009). "The proapoptotic dp5 gene is a direct target of the MLK-JNK-c-Jun pathway in sympathetic neurons." Nucleic Acids Res **37**(9): 3044-60.

Traynelis, S. F. and S. G. Cull-Candy (1990). "Proton inhibition of N-methyl-D-aspartate receptors in cerebellar neurons." Nature **345**(6273): 347-50.

Traynelis, S. F., L. P. Wollmuth, C. J. McBain, F. S. Menniti, K. M. Vance, K. K. Ogden, K. B. Hansen, H. Yuan, S. J. Myers and R. Dingledine (2010). "Glutamate receptor ion channels: structure, regulation, and function." Pharmacol Rev **62**(3): 405-96.

Trichonas, G., Y. Murakami, A. Thanos, Y. Morizane, M. Kayama, C. M. Debouck, T. Hisatomi, J. W. Miller and D. G. Vavvas (2010). "Receptor interacting protein kinases mediate retinal detachment-induced photoreceptor necrosis and compensate for inhibition of apoptosis." Proc Natl Acad Sci U S A **107**(50): 21695-700.

Tschopp, J., M. Irmler and M. Thome (1998). "Inhibition of fas death signals by FLIPs." Curr Opin Immunol **10**(5): 552-8.

Tu, W., X. Xu, L. Peng, X. Zhong, W. Zhang, M. M. Soundarapandian, C. Balel, M. Wang, N. Jia, F. Lew, S. L. Chan, Y. Chen and Y. Lu (2010). "DAPK1 interaction with NMDA receptor NR2B subunits mediates brain damage in stroke." Cell **140**(2): 222-34.

Tuttolomondo, A., D. Di Raimondo, R. Pecoraro, V. Arnao, A. Pinto and G. Licata (2012). "Inflammation in ischemic stroke subtypes." Curr Pharm Des **18**(28): 4289-310.

Tymianski, M. and C. H. Tator (1996). "Normal and abnormal calcium homeostasis in neurons: a basis for the pathophysiology of traumatic and ischemic central nervous system injury." Neurosurgery **38**(6): 1176-95.

Uhlik, M. T., A. N. Abell, N. L. Johnson, W. Sun, B. D. Cuevas, K. E. Lobel-Rice, E. A. Horne, M. L. Dell'Acqua and G. L. Johnson (2003). "Rac-MEKK3-MKK3 scaffolding for p38 MAPK activation during hyperosmotic shock." Nat Cell Biol **5**(12): 1104-10.

Upton, J. W., W. J. Kaiser and E. S. Mocarski (2010). "Virus inhibition of RIP3-dependent necrosis." Cell Host Microbe **7**(4): 302-13.

Van Herreweghe, F., N. Festjens, W. Declercq and P. Vandenabeele (2010). "Tumor necrosis factor-mediated cell death: to break or to burst, that's the question." Cell Mol Life Sci **67**(10): 1567-79.

van Loo, G., M. van Gurp, B. Depuydt, S. M. Srinivasula, I. Rodriguez, E. S. Alnemri, K. Gevaert, J. Vandekerckhove, W. Declercq and P. Vandenabeele (2002). "The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity." Cell Death Differ **9**(1): 20-6.

Vanden Berghe, T., G. van Loo, X. Saelens, M. Van Gurp, G. Brouckaert, M. Kalai, W. Declercq and P. Vandenabeele (2004). "Differential signaling to apoptotic and necrotic cell death by Fas-associated death domain protein FADD." J Biol Chem **279**(9): 7925-33.

Vandenabeele, P., L. Galluzzi, T. Vanden Berghe and G. Kroemer (2010). "Molecular mechanisms of necroptosis: an ordered cellular explosion." Nat Rev Mol Cell Biol **11**(10): 700-14.

Vanlangenakker, N., M. J. Bertrand, P. Bogaert, P. Vandenabeele and T. Vanden Berghe (2011a). "TNF-induced necroptosis in L929 cells is tightly regulated by multiple TNFR1 complex I and II members." Cell Death Dis **2**: e230.

Vanlangenakker, N., T. Vanden Berghe, P. Bogaert, B. Laukens, K. Zobel, K. Deshayes, D. Vucic, S. Fulda, P. Vandenabeele and M. J. Bertrand (2011b). "cIAP1 and TAK1 protect cells from TNF-induced necrosis by preventing RIP1/RIP3-dependent reactive oxygen species production." Cell Death Differ **18**(4): 656-65.

Varfolomeev, E., T. Goncharov, A. V. Fedorova, J. N. Dynek, K. Zobel, K. Deshayes, W. J. Fairbrother and D. Vucic (2008). "c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation." J Biol Chem **283**(36): 24295-9.

Ventura, J. J., P. Cogswell, R. A. Flavell, A. S. Baldwin, Jr. and R. J. Davis (2004). "JNK potentiates TNF-stimulated necrosis by increasing the production of cytotoxic reactive oxygen species." Genes Dev **18**(23): 2905-15.

Ventura, J. J., A. Hubner, C. Zhang, R. A. Flavell, K. M. Shokat and R. J. Davis (2006). "Chemical genetic analysis of the time course of signal transduction by JNK." Mol Cell **21**(5): 701-10.

Vercammen, D., R. Beyaert, G. Denecker, V. Goossens, G. Van Loo, W. Declercq, J. Grooten, W. Fiers and P. Vandenabeele (1998). "Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor." J Exp Med **187**(9): 1477-85.

Verhelst, K., I. Carpentier, M. Kreike, L. Meloni, L. Verstrepen, T. Kensche, I. Dikic and R. Beyaert (2012). "A20 inhibits LUBAC-mediated NF-kappaB activation by binding linear polyubiquitin chains via its zinc finger 7." EMBO J **31**(19): 3845-55.

Vieira, M., J. Fernandes, A. Burgeiro, G. M. Thomas, R. L. Huganir, C. B. Duarte, A. L. Carvalho and A. E. Santos (2010). "Excitotoxicity through Ca2+-permeable AMPA receptors requires Ca2+-dependent JNK activation." Neurobiol Dis **40**(3): 645-55.

Vissel, B., J. J. Krupp, S. F. Heinemann and G. L. Westbrook (2001). "A use-dependent tyrosine dephosphorylation of NMDA receptors is independent of ion flux." Nat Neurosci **4**(6): 587-96.

Vosler, P. S., Y. Gao, C. S. Brennan, A. Yanagiya, Y. Gan, G. Cao, F. Zhang, S. J. Morley, N. Sonenberg, M. V. Bennett and J. Chen (2011). "Ischemia-induced calpain activation causes eukaryotic (translation) initiation factor 4G1 (eIF4GI) degradation, protein synthesis inhibition, and neuronal death." Proc Natl Acad Sci U S A **108**(44): 18102-7.

Waetzig, G. H., P. Rosenstiel, S. Nikolaus, D. Seegert and S. Schreiber (2003). "Differential p38 mitogen-activated protein kinase target phosphorylation in responders and nonresponders to infliximab." Gastroenterology **125**(2): 633-4; author reply 635-6.

Waetzig, V., Y. Zhao and T. Herdegen (2006). "The bright side of JNKs-Multitalented mediators in neuronal sprouting, brain development and nerve fiber regeneration." Prog Neurobiol **80**(2): 84-97.

Wahl, A. S., B. Buchthal, F. Rode, S. F. Bomholt, H. E. Freitag, G. E. Hardingham, L. C. Ronn and H. Bading (2009). "Hypoxic/ischemic conditions induce expression of the putative pro-death gene Clca1 via activation of extrasynaptic N-methyl-D-aspartate receptors." Neuroscience **158**(1): 344-52.

Wan, Y. Y., H. Chi, M. Xie, M. D. Schneider and R. A. Flavell (2006). "The kinase TAK1 integrates antigen and cytokine receptor signaling for T cell development, survival and function." Nat Immunol **7**(8): 851-8.

Wang, C. C., R. G. Held, S. C. Chang, L. Yang, E. Delpire, A. Ghosh and B. J. Hall (2011). "A critical role for GluN2B-containing NMDA receptors in cortical development and function." Neuron **72**(5): 789-805.

Wang, C. Y., M. W. Mayo, R. G. Komeluk, D. V. Goeddel and A. S. Baldwin, Jr. (1998a). "NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation." Science **281**: 1680-3.

Wang, D., Q. Fu, Y. Zhou, B. Xu, Q. Shi, B. Igwe, L. Matt, J. W. Hell, E. V. Wisely, S. Oddo and Y. K. Xiang (2013). "beta2 adrenergic receptor, protein kinase A (PKA) and c-Jun N-terminal kinase (JNK) signaling pathways mediate tau pathology in Alzheimer disease models." J Biol Chem **288**(15): 10298-307.

Wang, J., S. Liu, Y. Fu, J. H. Wang and Y. Lu (2003). "Cdk5 activation induces hippocampal CA1 cell death by directly phosphorylating NMDA receptors." Nat Neurosci **6**(10): 1039-47.

Wang, W. W., S. Q. Hu, C. Li, C. Zhou, S. H. Qi and G. Y. Zhang (2010). "Transduced PDZ1 domain of PSD-95 decreases Src phosphorylation and increases nNOS (Ser847) phosphorylation contributing to neuroprotection after cerebral ischemia." Brain Res **1328**: 162-70.

Wang, X. S., K. Diener, T. H. Tan and Z. Yao (1998b). "MAPKKK6, a novel mitogen-activated protein kinase kinase kinase, that associates with MAPKKK5." Biochem Biophys Res Commun **253**(1): 33-7.

Wang, Y. and Z. H. Qin (2010). "Molecular and cellular mechanisms of excitotoxic neuronal death." Apoptosis **15**(11): 1382-402.

Wang, Y. Q., L. Wang, M. Y. Zhang, T. Wang, H. J. Bao, W. L. Liu, D. K. Dai, L. Zhang, P. Chang, W. W. Dong, X. P. Chen and L. Y. Tao (2012a). "Necrostatin-1 suppresses autophagy and apoptosis in mice traumatic brain injury model." Neurochem Res **37**(9): 1849-58.

Wang, Z., H. Jiang, S. Chen, F. Du and X. Wang (2012b). "The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways." Cell **148**(1-2): 228-43.

Watanabe, M., Y. Inoue, K. Sakimura and M. Mishina (1992). "Developmental changes in distribution of NMDA receptor channel subunit mRNAs." Neuroreport **3**(12): 1138-40.

Watson, A., A. Eilers, D. Lallemand, J. Kyriakis, L. L. Rubin and J. Ham (1998). "Phosphorylation of c-Jun is necessary for apoptosis induced by survival signal withdrawal in cerebellar granule neurons." J Neurosci **18**(2): 751-62.

Weinlich, R., C. P. Dillon and D. R. Green (2011). "Ripped to death." Trends Cell Biol **21**(11): 630-7.

Weitlauf, C., Y. Honse, Y. P. Auberson, M. Mishina, D. M. Lovinger and D. G. Winder (2005). "Activation of NR2A-containing NMDA receptors is not obligatory for NMDA receptor-dependent long-term potentiation." J Neurosci **25**(37): 8386-90.

Wellbrock, C., M. Karasarides and R. Marais (2004). "The RAF proteins take centre stage." Nat Rev Mol Cell Biol **5**(11): 875-85.

Welz, P. S., A. Wullaert, K. Vlantis, V. Kondylis, V. Fernandez-Majada, M. Ermolaeva, P. Kirsch, A. Sterner-Kock, G. van Loo and M. Pasparakis (2011). "FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation." Nature **477**(7364): 330-4.

Wenthold, R. J., K. Prybylowski, S. Standley, N. Sans and R. S. Petralia (2003). "Trafficking of NMDA receptors." Annu Rev Pharmacol Toxicol **43**: 335-58.

Wertz, I. E., K. M. O'Rourke, H. Zhou, M. Eby, L. Aravind, S. Seshagiri, P. Wu, C. Wiesmann, R. Baker, D. L. Boone, A. Ma, E. V. Koonin and V. M. Dixit (2004). "De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling." Nature **430**(7000): 694-9.

Weston, C. R. and R. J. Davis (2002). "The JNK signal transduction pathway." Curr Opin Genet Dev **12**(1): 14-21.

Weston, C. R. and R. J. Davis (2007). "The JNK signal transduction pathway." Curr Opin Cell Biol **19**(2): 142-9.

White, B. J., S. Tarabishy, V. R. Venna, B. Manwani, S. Benashski, L. D. McCullough and J. Li (2012). "Protection from cerebral ischemia by inhibition of TGFbeta-activated kinase." Exp Neurol **237**(1): 238-45.

Whitfield, J., S. J. Neame, L. Paquet, O. Bernard and J. Ham (2001). "Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release." Neuron **29**(3): 629-43.

Whitmarsh, A. J., J. Cavanagh, C. Tournier, J. Yasuda and R. J. Davis (1998). "A mammalian scaffold complex that selectively mediates MAP kinase activation." Science **281**(5383): 1671-4.

Wilde, G. J., A. K. Pringle, L. E. Sundstrom, D. A. Mann and F. Iannotti (2000). "Attenuation and augmentation of ischaemia-related neuronal death by tumour necrosis factor-alpha in vitro." Eur J Neurosci **12**(11): 3863-70.

Wilhelm, M., Z. Xu, N. V. Kukekov, S. Gire and L. A. Greene (2007). "Proapoptotic Nix activates the JNK pathway by interacting with POSH and mediates death in a Parkinson disease model." J Biol Chem **282**(2): 1288-95.

Williams, K. (1993). "Ifenprodil discriminates subtypes of the N-methyl-D-aspartate receptor: selectivity and mechanisms at recombinant heteromeric receptors." Mol Pharmacol **44**(4): 851-9.

Willoughby, E. A. and M. K. Collins (2005). "Dynamic interaction between the dual specificity phosphatase MKP7 and the JNK3 scaffold protein beta-arrestin 2." J Biol Chem **280**(27): 25651-8.

Willoughby, E. A., G. R. Perkins, M. K. Collins and A. J. Whitmarsh (2003). "The JNK-interacting protein-1 scaffold protein targets MAPK phosphatase-7 to dephosphorylate JNK." J Biol Chem **278**(12): 10731-6.

Won, S. J., D. Y. Kim and B. J. Gwag (2002). "Cellular and molecular pathways of ischemic neuronal death." J Biochem Mol Biol **35**(1): 67-86.

Wong, W. W., I. E. Gentle, U. Nachbur, H. Anderton, D. L. Vaux and J. Silke (2010). "RIPK1 is not essential for TNFR1-induced activation of NF-kappaB." Cell Death Differ **17**(3): 482-7.

Wright, A., W. W. Reiley, M. Chang, W. Jin, A. J. Lee, M. Zhang and S. C. Sun (2007). "Regulation of early wave of germ cell apoptosis and spermatogenesis by deubiquitinating enzyme CYLD." Dev Cell **13**(5): 705-16.

Wu, C. J., D. B. Conze, T. Li, S. M. Srinivasula and J. D. Ashwell (2006a). "Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]." Nat Cell Biol **8**(4): 398-406.

Wu, J. J., R. J. Roth, E. J. Anderson, E. G. Hong, M. K. Lee, C. S. Choi, P. D. Neufer, G. I. Shulman, J. K. Kim and A. M. Bennett (2006b). "Mice lacking MAP kinase phosphatase-1 have enhanced MAP kinase activity and resistance to diet-induced obesity." Cell Metab **4**(1): 61-73.

Wyllie, D. J., M. R. Livesey and G. E. Hardingham (2013). "Influence of GluN2 subunit identity on NMDA receptor function." Neuropharmacology.

Xu, J., P. Kurup, Y. Zhang, S. M. Goebel-Goody, P. H. Wu, A. H. Hawasli, M. L. Baum, J. A. Bibb and P. J. Lombroso (2009). "Extrasynaptic NMDA receptors couple preferentially to excitotoxicity via calpain-mediated cleavage of STEP." J Neurosci **29**(29): 9330-43.

Xu, X., K. W. Chua, C. C. Chua, C. F. Liu, R. C. Hamdy and B. H. Chua (2010). "Synergistic protective effects of humanin and necrostatin-1 on hypoxia and ischemia/reperfusion injury." Brain Res **1355**: 189-94.

Xu, Y., S. Huang, Z. G. Liu and J. Han (2006). "Poly(ADP-ribose) polymerase-1 signaling to mitochondria in necrotic cell death requires RIP1/TRAF2-mediated JNK1 activation." J Biol Chem **281**(13): 8788-95.

Xu, Z., N. V. Kukekov and L. A. Greene (2003). "POSH acts as a scaffold for a multiprotein complex that mediates JNK activation in apoptosis." EMBO J **22**(2): 252-61.

Xu, Z., N. V. Kukekov and L. A. Greene (2005). "Regulation of apoptotic c-Jun N-terminal kinase signaling by a stabilization-based feed-forward loop." Mol Cell Biol **25**(22): 9949-59.

Xu, Z., A. C. Maroney, P. Dobrzanski, N. V. Kukekov and L. A. Greene (2001). "The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis." Mol Cell Biol **21**(14): 4713-24.

Xu, Z., O. L. Tavares-Sanchez, Q. Li, J. Fernando, C. M. Rodriguez, E. J. Studer, W. M. Pandak, P. B. Hylemon and G. Gil (2007). "Activation of bile acid biosynthesis by the p38 mitogen-activated protein kinase (MAPK): hepatocyte nuclear factor-4alpha phosphorylation by the p38 MAPK is required for cholesterol 7alpha-hydroxylase expression." J Biol Chem **282**(34): 24607-14.

Yamamoto, K., H. Ichijo and S. J. Korsmeyer (1999). "BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M." Mol Cell Biol **19**(12): 8469-78.

Yamanaka, K., Y. Saito, T. Yamamori, Y. Urano and N. Noguchi (2011). "24(S)-hydroxycholesterol induces neuronal cell death through necroptosis, a form of programmed necrosis." J Biol Chem **286**(28): 24666-73.

Yang, D. D., C. Y. Kuan, A. J. Whitmarsh, M. Rincon, T. S. Zheng, R. J. Davis, P. Rakic and R. A. Flavell (1997). "Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene." Nature **389**(6653): 865-70.

Yang, Q. H. and C. Du (2004). "Smac/DIABLO selectively reduces the levels of c-IAP1 and c-IAP2 but not that of XIAP and livin in HeLa cells." J Biol Chem **279**(17): 16963-70.

Yang, Y., W. Hu, S. Feng, J. Ma and M. Wu (2005). "RIP3 beta and RIP3 gamma, two novel splice variants of receptor-interacting protein 3 (RIP3), downregulate RIP3-induced apoptosis." Biochem Biophys Res Commun **332**(1): 181-7.

Yang, Y., J. Ma, Y. Chen and M. Wu (2004). "Nucleocytoplasmic shuttling of receptor-interacting protein 3 (RIP3): identification of novel nuclear export and import signals in RIP3." J Biol Chem **279**(37): 38820-9.

Yao, R., M. Yoshihara and H. Osada (1997). "Specific activation of a c-Jun NH2-terminal kinase isoform and induction of neurite outgrowth in PC-12 cells by staurosporine." J Biol Chem **272**(29): 18261-6.

Yao, Y., C. B. Harrison, P. L. Freddolino, K. Schulten and M. L. Mayer (2008). "Molecular mechanism of ligand recognition by NR3 subtype glutamate receptors." EMBO J **27**(15): 2158-70.

Yao, Z., G. Zhou, X. S. Wang, A. Brown, K. Diener, H. Gan and T. H. Tan (1999). "A novel human STE20-related protein kinase, HGK, that specifically activates the c-Jun N-terminal kinase signaling pathway." J Biol Chem **274**(4): 2118-25.

Yasuda, J., A. J. Whitmarsh, J. Cavanagh, M. Sharma and R. J. Davis (1999). "The JIP group of mitogen-activated protein kinase scaffold proteins." Mol Cell Biol **19**(10): 7245-54.

Yi, Z., R. S. Petralia, Z. Fu, C. C. Swanwick, Y. X. Wang, K. Prybylowski, N. Sans, S. Vicini and R. J. Wenthold (2007). "The role of the PDZ protein GIPC in regulating NMDA receptor trafficking." J Neurosci **27**(43): 11663-75.

Yin, F., D. Bruemmer, F. Blaschke, W. A. Hsueh, R. E. Law and A. J. Herle (2004). "Signaling pathways involved in induction of GADD45 gene expression and apoptosis by troglitazone in human MCF-7 breast carcinoma cells." Oncogene **23**(26): 4614-23.

Yoon, S. and R. Seger (2006). "The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions." Growth Factors **24**(1): 21-44.

Yoshioka, K. (2004). "Scaffold proteins in mammalian MAP kinase cascades." J Biochem **135**(6): 657-61.

You, Z., S. I. Savitz, J. Yang, A. Degterev, J. Yuan, G. D. Cuny, M. A. Moskowitz and M. J. Whalen (2008). "Necrostatin-1 reduces histopathology and improves functional outcome after controlled cortical impact in mice." J Cereb Blood Flow Metab **28**(9): 1564-73.

Yu, C. Z., C. Li, D. S. Pei, Y. Y. Zong, Q. Shi, X. R. Wen, Q. H. Guan, D. Hang, X. Y. Hou and G. Y. Zhang (2009). "Neuroprotection against transient focal cerebral ischemia and oxygen-glucose deprivation by interference with GluR6-PSD95 protein interaction." Neurochem Res **34**(11): 2008-21.

Yu, L., A. Alva, H. Su, P. Dutt, E. Freundt, S. Welsh, E. H. Baehrecke and M. J. Lenardo (2004). "Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8." Science **304**(5676): 1500-2.

Yu, X. J., C. Y. Li, H. Y. Dai, D. X. Cai, K. Y. Wang, Y. H. Xu, L. M. Chen and C. L. Zhou (2007). "Expression and localization of the activated mitogen-activated protein kinase in lesional psoriatic skin." Exp Mol Pathol **83**(3): 413-8.

Zablocka, B., J. Dluzniewska, H. Zajac and K. Domanska-Janik (2003). "Opposite reaction of ERK and JNK in ischemia vulnerable and resistant regions of hippocampus: involvement of mitochondria." Brain Res Mol Brain Res **110**(2): 245-52.

Zhang, D. W., J. Shao, J. Lin, N. Zhang, B. J. Lu, S. C. Lin, M. Q. Dong and J. Han (2009a). "RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis." Science **325**(5938): 332-6.

Zhang, D. W., M. Zheng, J. Zhao, Y. Y. Li, Z. Huang, Z. Li and J. Han (2011a). "Multiple death pathways in TNF-treated fibroblasts: RIP3- and RIP1-dependent and independent routes." Cell Res **21**(2): 368-71.

Zhang, F., C. Li, R. Wang, D. Han, Q. G. Zhang, C. Zhou, H. M. Yu and G. Y. Zhang (2007a). "Activation of GABA receptors attenuates neuronal apoptosis through inhibiting the tyrosine phosphorylation of NR2A by Src after cerebral ischemia and reperfusion." Neuroscience **150**(4): 938-49.

Zhang, J. and J. S. Diamond (2009). "Subunit- and pathway-specific localization of NMDA receptors and scaffolding proteins at ganglion cell synapses in rat retina." J Neurosci **29**(13): 4274-86.

Zhang, L., L. Y. Dong, Y. J. Li, Z. Hong and W. S. Wei (2012). "The microRNA miR-181c controls microglia-mediated neuronal apoptosis by suppressing tumor necrosis factor." J Neuroinflammation **9**: 211.

Zhang, Q., G. Zhang, F. Meng and H. Tian (2003). "Biphasic activation of apoptosis signal-regulating kinase 1-stress-activated protein kinase 1-c-Jun N-terminal protein kinase pathway is selectively mediated by Ca2+-permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors involving oxidative stress following brain ischemia in rat hippocampus." Neurosci Lett **337**(1): 51-5.

Zhang, S. J., B. Buchthal, D. Lau, S. Hayer, O. Dick, M. Schwaninger, R. Veltkamp, M. Zou, U. Weiss and H. Bading (2011b). "A signaling cascade of nuclear calcium-CREB-ATF3 activated by synaptic NMDA receptors defines a gene repression module that protects against extrasynaptic NMDA receptor-induced neuronal cell death and ischemic brain damage." J Neurosci **31**(13): 4978-90.

Zhang, S. J., M. N. Steijaert, D. Lau, G. Schutz, C. Delucinge-Vivier, P. Descombes and H. Bading (2007b). "Decoding NMDA receptor signaling: identification of genomic programs specifying neuronal survival and death." Neuron **53**(4): 549-62.

Zhang, S. J., M. Zou, L. Lu, D. Lau, D. A. Ditzel, C. Delucinge-Vivier, Y. Aso, P. Descombes and H. Bading (2009b). "Nuclear calcium signaling controls

expression of a large gene pool: identification of a gene program for acquired neuroprotection induced by synaptic activity." PLoS Genet **5**(8): e1000604.

Zhang, Y., J. N. Blattman, N. J. Kennedy, J. Duong, T. Nguyen, Y. Wang, R. J. Davis, P. D. Greenberg, R. A. Flavell and C. Dong (2004). "Regulation of innate and adaptive immune responses by MAP kinase phosphatase 5." Nature **430**(7001): 793-7.

Zhang, Y., L. Zhou and C. A. Miller (1998). "A splicing variant of a death domain protein that is regulated by a mitogen-activated kinase is a substrate for c-Jun N-terminal kinase in the human central nervous system." Proc Natl Acad Sci U S A **95**(5): 2586-91.

Zhao, J., S. Jitkaew, Z. Cai, S. Choksi, Q. Li, J. Luo and Z. G. Liu (2012). "Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis." Proc Natl Acad Sci U S A **109**(14): 5322-7.

Zhao, Q., X. Wang, L. D. Nelin, Y. Yao, R. Matta, M. E. Manson, R. S. Baliga, X. Meng, C. V. Smith, J. A. Bauer, C. H. Chang and Y. Liu (2006). "MAP kinase phosphatase 1 controls innate immune responses and suppresses endotoxic shock." J Exp Med **203**(1): 131-40.

Zheng, C. Y., G. K. Seabold, M. Horak and R. S. Petralia (2011). "MAGUKs, synaptic development, and synaptic plasticity." Neuroscientist **17**(5): 493-512.

Zhou, L., F. Li, H. B. Xu, C. X. Luo, H. Y. Wu, M. M. Zhu, W. Lu, X. Ji, Q. G. Zhou and D. Y. Zhu (2010). "Treatment of cerebral ischemia by disrupting ischemia-induced interaction of nNOS with PSD-95." Nat Med **16**(12): 1439-43.

Zhou, M., R. Dominguez and M. Baudry (2007). "Superoxide dismutase/catalase mimetics but not MAP kinase inhibitors are neuroprotective against oxygen/glucose deprivation-induced neuronal death in hippocampus." J Neurochem **103**(6): 2212-23.

Zhou, X., Q. Ding, Z. Chen, H. Yun and H. Wang (2013). "Involvement of GluN2A and GluN2B in synaptic and extrasynaptic NMDA receptor function and neuronal excitotoxicity." J Biol Chem.

Zhu, Q. J., Y. Xu, C. P. Du and X. Y. Hou (2012). "SUMOylation of the kainate receptor subunit GluK2 contributes to the activation of the MLK3-JNK3 pathway following kainate stimulation." FEBS Lett **586**(9): 1259-64.

Zhu, R. L., S. H. Graham, J. Jin, R. A. Stetler, R. P. Simon and J. Chen (1997). "Kainate induces the expression of the DNA damage-inducible gene, GADD45, in the rat brain." Neuroscience **81**(3): 707-20.

Zukin, R. S., T. Jover, H. Yokota, A. Calderone, M. Simionescu and C. G. Lau (2004). Molecular and Cellular Mechanisms of Ischemia-Induced Neuronal Death. Stroke: Pathophysiology, Diagnosis, and Management J. P. Mohr, D. W. Choi, J. C. Grotta, B. Weir and P. A. Wolf. Philadelphia, Elsevier, Inc**:** 829-854.