UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA DE LISBOA

AMYLOID β PEPTIDE AND p63: TWO PRIME TRIGGERS OF NEURAL APOPTOSIS AND DIFFERENTIATION

Maria Benedita Pereira de Vasconcelos Fonseca

Doutoramento em Ciências Biomédicas (Neurociências), apresentada à Universidade de Lisboa através da Faculdade de Medicina

2013

UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA DE LISBOA

AMYLOID β PEPTIDE AND p63: TWO PRIME TRIGGERS OF NEURAL APOPTOSIS AND DIFFERENTIATION

Maria Benedita Pereira de Vasconcelos Fonseca

Doutoramento em Ciências Biomédicas (Neurociências), apresentada à Universidade de Lisboa através da Faculdade de Medicina

Supervisor: Cecília M. P. Rodrigues, Ph.D.
Co-supervisor: Ana Sebastião, Ph.D.

O presente documento é da exclusiva responsabilidade do seu autor, não cabendo qualquer responsabilidade à Faculdade de Medicina de Lisboa pelos conteúdos nele apresentados

The studies presented in this thesis were performed at the Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL) and Department of Biochemistry and Human Biology, Faculty of Pharmacy, University of Lisbon, under the supervision of Professor Cecília M. P. Rodrigues.

Maria Benedita Pereira de Vasconcelos Fonseca was the recipient of a Ph.D. fellowship (SFRH/BD/43959/2008) from Fundação para a Ciência e Tecnologia (FCT), Lisbon, Portugal. This work was supported by grants PTDC/SAU-FCF/67912/2006, PTDC/SAU-NMC/117877/2010 and Pest-OE/SAU/UI4013/2011 from FCT and FEDER.

De acordo com o disposto no ponto 1 do artigo nº 41 do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, deliberação nº 93/2006, publicada em Diário da República – II Série nº 153 – 5 de Julho de 2003, o Autor desta dissertação declara que participou na conceção e execução do trabalho experimental, interpretação dos resultados obtidos e redação dos manuscritos.

A impressão desta dissertação foi aprovada pela Comissão Coordenadora do Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 18 de junho de 2013.

Agradecimentos

Foi uma longa viagem... Dedico este espaço àqueles que me acompanharam ao longo desta jornada, que me ajudaram e apoiaram a tornar um sonho realidade. Naturalmente, não conseguirei nomear aqui todas as pessoas, mas há no entanto alguns a quem não posso deixar de manifestar o meu apreço e agradecimento sinceros.

Muito especialmente, agradeço à minha orientadora Professora Cecília Rodrigues, um exemplo que para sempre levarei comigo, como pessoa e como profissional. Muito obrigada por me ter orientado, por ter acreditado em mim, por me incentivar e apoiar sempre que precisei, pela sua total disponibilidade, paciência e compreensão.

Agradeço à Professora Ana Sebastião, minha coorientadora, pelo tempo dispendido na análise da tese.

Agradeço à Filipa Nunes, pela sua excelente orientação, por todo o saber que me transmitiu, sempre com muita, muita paciência, dedicação e um sorriso. Obrigada amiga, do fundo do coração. Tive também a oportunidade de ser orientada pela Susana Solá, a quem também quero agradecer a sua exaustiva dedicação e atenção prestadas ao meu projeto, mesmo quando a sua agenda parecia não ter espaço para mais nada. Obrigada Susana, por todo o teu apoio.

Aos meus colegas e ex-colegas do laboratório e sempre amigos, o meu muitíssimo obrigada por me terem acompanhado e ajudado ao longo desta "caminhada". Agradeço a força e o incentivo constantes, o espírito de grupo e a amizade de todos os colegas. Ao Duarte, um beijinho especial pela nossa amizade, por ter sempre tempo para me escutar, para esclarecer uma dúvida, para ensinar, para as nossas conversas sobre ginásio, para dar um abracinho reconfortante, sempre de uma forma tão sincera. À Joana Xavier, obrigada pelos nossos momentos de desabafo, pela sua ajuda incondicional no laboratório e não só, e pela sua pro-atividade, um exemplo a seguir. Ao André também um agradecimento especial, por ser uma pessoa com um coração tão bom, tão espontâneo e ser tão sorridente. Aqui fica a promessa de enviar-lhe de vez em quando uma caixinha de quindins. À Ana Luísa, o meu reconhecimento por ser sempre tão prestável para todos os colegas do grupo. Obrigada Ana também pela tua ajuda preciosa em momentos de maior aflição no laboratório. Aos meus colegas mais novos,

Pedro Rodrigues, Pedro Dionísio, Diane, Sofia e Marta, um beijinho grande, vocês trouxeram energia nova para o laboratório. Tenho um grande carinho por vocês e desejo-vos grandes sucessos. À Rita, Rui, Joana Amaral, Pedro Borralho, Daniela, Ricardo e Márcia, os meus colegas que me receberam tão bem quando entrei no grupo e me viram "crescer" ao longo destes anos, um agradecimento sincero pelo vosso apoio incondicional, pela atenção dispensada, pelas críticas e opiniões e pelo convívio diário, sempre muito bem dispostos. Agradeço igualmente às Professoras Elsa Rodrigues, Maria João Gama, Margarida Castro Caldas, bem como à Andreia, Inês e ao Miguel, pela sempre boa disposiçao que nos contagia. Manifesto aqui também a minha gratidão à Professora Isabel Bettencourt e a todas as pessoas do iMed.UL que tive oportunidade de conhecer e com quem partilhei os meus momentos, bons e menos bons. À Deolinda, um beijinho com muito carinho, pela seu sorriso diário, pela sua simplicidade e vontade de ajudar.

Aos meus queridos amigos Heloísa, Miguel, Margarida e Vítor, não há palavras para descrever o quão gosto de vocês e o quão importantes são para mim. Eles são como família, os meus queridos companheiros de treino, de sofrimento, de alegrias, de "beca-beca". Estou eternamente grata a Deus por eles fazerem parte da minha vida.

Aos meus pais e ao meu irmão João, de quem tenho tantas saudades, um beijinho com muito amor e um muito obrigada pelo incentivo permanente e pela compreensão da distância que o trabalho me impediu algumas vezes de minimizar. Um obrigada também aos meus avós, primos e tios, sempre tão presentes. À Adelina, a minha segunda mãe, e ao Rui, Sofia, Leonor e Guilherme, agradeço do fundo do coração todo o carinho e amor que sempre me deram de uma forma tão simples e sincera.

Por fim, um agradecimento muito especial para um ser muito especial. Diogo, a ti dedico esta tese, por todo o amor, pelo suporte emocional, por seres tantas vezes um pai ou uma mãe para mim, para além de namorado, por seres a pessoa que mais admiro neste mundo. O teu olhar e o teu sorriso rasgado e tão teu foram muitas vezes o meu alento em dias menos claros. Eyes do not lie...!!!

Table of Contents

Figures

Resumo

A doença de Alzheimer (AD) é uma doença neurodegenerativa caracterizada pela ocorrência de perturbações na função sináptica e perda massiva de neurónios nas regiões cerebrais relacionadas com as funções cognitivas, como o hipocampo e o córtex cerebral. A deposição do péptido β amilóide (Aβ) na AD foi desde cedo reportada como sendo um aspeto crucial nos fenómenos patológicos da doença, nomeadamente na formação de placas senis. Vários estudos mostram que o stress oxidativo, a inflamação, a perturbação dos fluxos de cálcio e a morte celular programada estão envolvidos na toxicidade induzida pelo Aβ. Contudo, os mecanismos pelos quais o péptido Aβ desencadeia a morte neuronal não são, ainda, completamente conhecidos. Um melhor conhecimento desses mecanismos básicos, desencadeados pelo péptido Aβ, poderá constituir um importante contributo para retardar, ou até prevenir, a neurodegenerescência associada à AD. Por outro lado, alguns dos fatores associados ao processo apoptótico podem também modular a proliferação e a diferenciação. Os mecanismos regulatórios desta tomada de decisão, quanto ao destino das células, dependem do balanço e da conjugação de sinais de sobrevivência e de morte celular. De facto, o nosso grupo demonstrou já que caspases, calpaínas, p53 e microRNAs associados à apoptose aceleram o processo de diferenciação neural. Além disso, foi recentemente sugerido que o péptido Aβ pode também regular a proliferação e diferenciação de células estaminais neurais (NSCs). Estas células são capazes de originar os principais tipos de células neurais, incluindo neurónios e células da glia, como os astrócitos e os oligodendrócitos. Em cada divisão celular, as NSCs podem seguir destinos celulares diferentes, nomeadamente continuar o processo proliferativo, ou iniciar a diferenciação. Assim, propusémo-nos identificar e caracterizar novos fatores e vias moleculares envolvidas na regulação da apoptose e da diferenciação neural, que sejam relevantes no contexto da AD.

Explorámos, inicialmente, mecanismos moleculares subjacentes à apoptose induzida pelo Aβ em células neuronais PC12 e em neurónios corticais de cultura primária. A incubação com o péptido Aβ resultou na estabilização da isoforma pro--apoptótica da proteína p63, pertencente à família p53, TAp63, bem como na degradação da isoforma anti-apoptótica, ΔNp63, através de um mecanismo dependente do proteossoma e de cinases de proteínas ativadas pelo mitogénio. Este efeito encontrou-se associado a um aumento dos níveis proteicos de c-Jun, um fator de transcrição envolvido na regulação do destino das células, já anteriormente reportado como sendo modulador da abundância de TAp63. Curiosamente, a pré-incubação das

células com um agente anti-apoptótico, o ácido tauro-ursodesoxicólico (TUDCA), reverteu o efeito do Aβ nas proteínas TAp63 e c-Jun. De igual forma, em células tratadas com compostos genotóxicos, como a cisplatina e a doxorrubina, verificámos um aumento da expressão de c-Jun, associado a uma redução dos níveis proteícos da isoforma ΔNp63. A expressão endógena e ectópica de ΔNp63 foi também dramaticamente reduzida aquando da sobrexpressão de c-Jun. Recorrendo à tecnologia de silencimento com siRNAs, silenciámos c-Jun em células tratadas com Aβ, o que preveniu a degradação da isoforma ΔNp63. Por outro lado, a sobreexpressão de c-Jun originou a redução dos níveis de ΔNp63. Por fim, verificámos que o tempo de semi-vida de ΔNp63 foi significantemente reduzido na presença de c-Jun, confirmando assim o papel de c-Jun na regulação da estabilidade de ΔNp63. Estes resultados indicaram que a abundância de ΔNp63, em resposta à morte apoptótica induzida pelo Aβ, é regulada por um mecanismo dependente de c-Jun.

De seguida, investigámos o envolvimento da proteína p63 na regulação do potencial de proliferação e diferenciação de NSCs. De facto, a proteína p53, homóloga de p63, foi recentemente descrita como fator limitante do potencial proliferativo das células estaminais, desempenhando mesmo um papel crucial durante a neurogénese. Por outro lado, foi também demonstrado que os efeitos pró-neurogénicos da proteína p53 resultam da sua interação com reguladores importantes da diferenciação neural, como a desmetilase JMJD3, específica para o resíduo de lisina-27 da histona 3. Considerando as semelhanças funcionais e estruturais entre as proteínas p53 e p63, investigámos uma possível interação molecular entre a desmetilase JMJD3 e a proteína p63 durante o processo de neurogénese. A proteína p63 já foi descrita como estando envolvida na diferenciação da epiderme e de outros tecidos epiteliais. Contudo, o seu envolvimento durante a diferenciação neural não foi inteiramente esclarecido. Os resultados obtidos demonstraram que a isoforma TAp63γ interage diretamente com a desmetilase JMJD3 e que esta regula os níveis de p63, estabelecendo-se um programa de expressão de marcadores neurais. De facto, a sobrexpressão de JMJD3 promoveu um aumento dos níveis de TAp63γ, de forma dependente da sua atividade de desmetilase. A sobrexpressão de TAp63γ aumentou os níveis do marcador de neurónios β-III tubulina, enquanto que o silenciamento de TAp63γ resultou em efeitos significativamente diferentes. Ensaios de imunoprecipitação permitiram-nos confirmar a interação direta entre TAp63γ e JMJD3, durante a diferenciação de NSCs, assim como a modulação dos níveis de metilação de TAp63γ pela JMJD3. Curiosamente, a JMJD3

regulou também a estabilidade e a distribuição celular da isoforma TAp63γ, bem como o processo de neurogénese regulado pela TAp63γ. Estes resultados clarificam o papel da proteína p63 na diferenciação das células precursoras neurais e demonstram que a isoforma TAp63γ é um alvo direto da JMJD3, sendo esta interação importante para a neurogénese.

Por fim, avaliámos a capacidade de diferentes péptidos Aβ modularem os processos de proliferação e diferenciação de NSCs, clarificando o papel da autofagia nos efeitos mediados pelos péptidos Aβ. De facto, embora considerados neurotóxicos, os péptidos Aβ estão também envolvidos em diversos eventos celulares não patogénicos. Por outro lado, a autofagia tem sido implicada na sobrevivência celular em resposta à toxicidade do Aβ, mas também em processos de diferenciação. Contudo, o seu papel concreto na diferenciação neural continua por esclarecer. Os resultados obtidos demonstraram que os péptidos $A\beta_{1-40}$ e $A\beta_{1-42}$ promovem preferencialmente a neurogénese e a gliogénese, respetivamente, enquanto que o péptido $A\beta_{25-35}$ não parece ter qualquer efeito nestes processos. Verificámos também que o papel do $A\beta_{1-40}$ na neurogénese é parcialmente dependente da sua função na proliferação de NSCs. Tal foi comprovado pelo aumento da fase S do ciclo celular e pelo aumento da marcação com bromodeoxiuridina em células expostas a $A\beta_{1-40}$. Além disso, o péptido $A\beta_{1-40}$ aumentou a atividade do promotor de Tuj1, validando assim os resultados anteriores que mostram o papel importante do péptido $A\beta_{1-40}$ na diferenciação neuronal. Observámos também que o processo autofágico parece estar envolvido nos efeitos mediados pelos diferentes péptidos Aβ nas NSCs, independentemente da produção de espécies reativas de oxigénio e da ocorrência de fenómenos apoptóticos. Inibindo a autogafia, através da adição do inibidor autofágico 3-metiladenina, verificou-se que os níveis proteicos de marcadores neuronais e gliais não aumentaram, mesmo na presença dos diferentes péptidos Aβ. Assim, estes resultados suportam e clarificam o papel de diferentes péptidos Aβ na regulação do destino celular das NSCs e sublinham a importância da autofagia no controlo deste processo.

Em conclusão, estes estudos contribuem com dados novos relativamente ao papel do péptido Aβ e da proteína p63 no controlo da decisão entre diferenciação e morte celular, os quais se poderão revelar úteis na modulação do destino celular na AD.

Palavras-chave: Ácido tauro-ursodesoxicólico; Apoptose; Autofagia; Células estaminais neurais; c-Jun; Neurogénese; p63; Péptido β amilóide

Abstract

Amyloid β (Aβ) peptide accumulation and apoptosis play an important role in the pathogenesis of Alzheimer's disease. However, the mechanisms by which Aβ mediates neuronal apoptosis are not completely elucidated. Mounting evidence also supports the involvement of specific apoptosis factors in neural stem cell (NSC) differentiation. Therefore, we set to identify and characterize new molecular pathways involved in Aβ-induced regulation of neural apoptosis and differentiation.

First, we further explored the molecular mechanisms of Aβ-induced neuronal death. We found that Aβ elicited stabilization of the pro-apoptotic isoform of p63, TAp63, which in turn was partially inhibited by tauroursodeoxycholic acid. In addition, in response to Aβ-induced apoptosis, the abundance of the anti-apoptotic isoform of p63, ΔNp63, was clearly reduced and tightly regulated in a c-Jun-dependent mechanism.

Next, we investigated whether apoptosis-associated molecule p63, member of the p53 family, might also be involved in differentiation of NSCs. We showed that TAp63γ interacted with the histone H3 lysine 27-specific demethylase JMJD3, a key regulator of neurogenesis, to redirect NSCs to differentiation, as an alternative to cell death. In addition, both TAp63γ and JMJD3 were coordinately regulated to establish a neural-specific gene expression pattern during NSC differentiation. We also found that JMJD3-demethylase activity was crucial in regulating TAp63γ half-life and nuclear accumulation.

Finally, we evaluated the ability of Aβ peptides to modulate NSC proliferation and differentiation, and investigated whether autophagy was involved in Aβ-induced alterations of NSC fate. We showed that $A\beta_{1-40}$ and $A\beta_{1-42}$ strongly enhanced neurogenesis and gliogenesis, respectively, while $A\beta_{25-35}$ did not influence NSC fate. Notably, autophagy was implicated in Aβ-mediated effects in NSCs, independently of reactive oxygen species production and apoptosis induction.

In conclusion, the work presented here provides additional insights into the molecular mechanisms implicated in Aβ- and p63-induced cell death signaling pathways, and extends our knowledge in considering these prime triggers of apoptosis as integral components of neural proliferation and differentiation.

Keywords: Amyloid β peptide; Apoptosis; Autophagy; c-Jun; Neural stem cells; Neurogenesis; p63; Tauroursodeoxycholic acid

Abbreviations

3-MA	3-methyladenine
AD	Alzheimer's disease
Aβ	amyloid β
AIF	apoptosis-inducing factor
AP	autophagosome
AP-1	activator protein-1
Apaf-1	apoptosis protease-activating factor 1
APP	amyloid precursor protein
ATG	autophagy-related
Bcl-2	B-cell lymphoma-2
Bcl-x_L	B-cell leukemia/lymphoma extra long
BH	Bcl-2 homology
BrdU	bromodeoxyuridine
C99	99-residue membrane-bound fragment
CHX	cycloheximide
CNS	central nervous system
Cyt *c*	cytochrome *c*
DG	dentate gyrus
EGF	epidermal growth factor
ESC	embryonic stem cells
FBS	fetal bovine serum
FGF	fibroblast growth factor
KO	knockout
NGF	nerve growth factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
IAP	inhibitor of apoptosis protein
Id1	inhibitor of differentiation 1
JMJD3	histone H3 lysine 27-specific demethylase

JNK	c-Jun N-terminal kinase
LC3	microtubule-associated protein light chain 3
LDH	lactate dehydrogenase
MAP2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
MeK	methylated lysine
miRNAs	microRNAs
MOMP	mitochondrial outer membrane permeabilisation
NeuN	neuronal nuclei
NG2	neuronal/glial 2
NPCs	Neuronal precursor cells
NSCs	neural stem cells
NTR	neurotrophin receptor
PBS	phosphate buffer saline
PC12	rat pheochromocytoma
PI3K	phosphatidylinositide 3 kinase
PS1	presenilin-1
PS2	presenilin-2
PTM	post-translational modifications
RG	radial glia
ROS	reactive oxygen species
SGZ	subgranular zone
siRNA	short-interference RNA
SVZ	subventricular zone
TNF	tumor necrosis factor
TrkA	tyrosine kinase receptor type 1
TUDCA	tauroursodeoxycholic acid
UDCA	ursodeoxycholic acid

Publications

The present thesis was mostly based on work that has been published in international peer-reviewed journals:

Fonseca MB, Nunes AF, Rodrigues CMP. c-Jun regulates the stability of anti-apoptotic ΔNp63 in amyloid β-induced apoptosis. *Journal of Alzheimer's Disease* 2012; 28 (3): 685-94.

Fonseca MB, Nunes AF, Solá S, Rodrigues CMP. p63 demethylation by JMJD3 modulates p63 stability and cellular distribution during neural stem cell differentiation. *Plos One* 2012; 7 (12): e52417.

Fonseca MB, Solá S, Xavier JM, Dionísio P, Rodrigues CMP. Amyloid β peptides promote autophagy-dependent differentiation of mouse neural stem cells. *Molecular Neurobiology* 2013; June [Epub ahead of print].

The following manuscripts have also been published during the course of the Ph.D. studies:

Viana RJ, **Fonseca MB**, Ramalho RM, Nunes AF, Rodrigues CM. Organelle stress sensors and cell death mechanisms in neurodegenerative diseases. *CNS Neurological Disorders - Drug Targets* 2010; 9 (6): 679-92.

Nunes AF, Amaral JD, Lo AC, **Fonseca MB**, Viana RJS, D'Hooge R, Rodrigues CMP. TUDCA, a bile acid, attenuates amyloid precursor protein processing and amyloid β deposition in APP/PS1 Mice. *Molecular Neurobiology* 2012; 45 (3): 440-54.

1. General Introduction

1.1 Neural Apoptosis

The term apoptosis was first introduced in 1972 by Kerr *et al.* to describe the morphological processes leading to controlled cellular destruction (Kerr et al., 1972). The apoptosis term has a Greek origin, meaning "falling off" or "dropping off", in analogy to leaves falling off trees or petals dropping off flowers. This analogy emphasizes that the death of living matter is a necessary part of the life cycle of organisms, being apoptosis an extremely coordinated and highly efficient form of cell death that plays a considerable role during physiological conditions, including development, differentiation, proliferation and function of the immune system; and in the removal of harmful cells.

During development of the central (CNS) and peripheral nervous systems, naturally occurring neuronal death is required during a specific time window to ensure the establishment of a correct match between the neuronal numbers and the size of the target tissue and for morphogenetic processes, such as neural tube closure (Oppenheim, 1991). The ultimate survival of any given neuron is determined by its capacity to sequester sufficient amounts of target-derived trophic factors, such as the nerve growth factor (NGF) (Kaplan and Miller, 2000). NGF binds to the neurotrophic tyrosine kinase receptor type 1 (TrkA) on the terminal axonal arbor and mediates cellular survival signaling. A second neurotrophin receptor (NTR), the $p75^{NTR}$, plays an antagonistic role in this system, promoting the elimination of the neurons that do not compete for target-derived NGF. The initial overproduction of neurons, followed by death of some, represents an adaptive process that provides enough neurons to form nerve cell circuits that are precisely matched to their functional specifications. Neuronal loss subsequent to this development window is still physiologically appropriate but may also contribute to neurological deficits. Notably, the cell death machinery has vital components of the maintenance and differentiation programs of adult stem cells (Aranha et al., 2009; Solá et al., 2012), which must be restrictively activated to assure differentiation efficiency, and carefully regulated to avoid cell loss. Therefore, it is not surprising that during differentiation, specific cellular changes occur in a similar manner to those observed during apoptosis (Chasis and Schrier, 1989). In addition, Biebl *et al.* (Biebl et al., 2000) have also reported that apoptosis may have an important regulatory function by eliminating

supernumerous cells from neurogenic regions, and may thus contribute to a self-renewal mechanism in the adult mammalian brain. Furthermore, several studies have demonstrated that neuronal apoptosis also occurs as a pathological event following excitotoxic, ischemic or traumatic nervous system injury. Finally, there is increasing evidence that apoptotic cell death is one of the mechanisms leading to neuronal loss in neurodegenerative diseases (Yuan and Yankner, 2000), including Huntington's disease, Parkinson's disease, and Alzheimer's disease (AD) (Lassmann et al., 1995; Ohyagi et al., 2005; Smale et al., 1995; Tang et al., 2005; Yao and Wood, 2009).

Deposition of amyloid β (Aβ) peptide in AD was earlier thought to initiate the pathological cascade of this disease, including the formation of senile plaques and neurofibrillary tangles (NFTs), neuronal loss, and dementia. Previous studies have demonstrated that Aβ causes neurotoxicity and neuronal cell apoptosis *in vivo* and *in vitro* (Alvarez et al., 2004; Cancino et al., 2008; Laurén et al., 2009). However, the mechanisms of Aβ-induced apoptosis remain to be clarified.

1.1.1 Apoptosis signaling pathways

Classic apoptosis consists in at least two phases, initiation and execution, which in turn results in the activation of cysteine-dependent aspartate directed proteases, termed caspases. Most morphological changes of apoptotic cells are caused by caspases, in a sequence of biochemical events. The death receptor (extrinsic) and mitochondrial (intrinsic) apoptosis pathways represent the canonical routes of caspase activation during the initiation phase (Kroemer et al., 2007).

1.1.1.1. Extrinsic pathway

Death receptors belong to the tumor necrosis factor (TNF) superfamily of cell surface receptors, and contain a cytoplasmic protein motif termed death domain that enables receptors to engage the cell apoptotic machinery. Caspase activation by the death receptor involves the binding of extracellular death ligands, such as Fas ligand (FasL) or TNFα to transmembrane death receptors, causing the recruitment and oligomerization of the adapter molecule Fas-associating death domain-containing protein (FADD) within the death-inducing signaling complex. Oligomerized FADD, in turn, binds the procaspase-8 and -10, causing their dimerization and activation

(Debatin and Krammer, 2004). Cytosolic and active caspase-8 and -10 then mediate the activation of the effector caspases-3, -6 and -7, causing further caspase activation events, and finally substrate proteolysis and cell death (Debatin and Krammer, 2004; Mattson, 2006).

1.1.1.2. Intrinsic pathway

Mitochondria play a key role in the regulation of apoptosis, collecting and integrating both pro- and anti-apoptotic signals, such as pro- and anti-apoptotic B-cell lymphoma-2 (Bcl-2) family proteins, p53, kinases, phosphatases, reactive oxygen species (ROS), calcium overload, viral proteins or toxins. Unlike in extrinsic apoptosis, caspase activation is not always a requirement for mitochondrial-induced apoptosis, although caspases accelerate the process. The mitochondrial pathway of apoptosis begins with mitochondrial outer membrane permeabilization (MOMP), and subsequent release of apoptotic factors from the mitochondria, including cytochrome *c* (cyt *c*), second mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein (IAP) binding protein with a low pI (Smac/DIABLO) and Omi stress-regulated endoprotease/high temperature requirement protein A2 (Omi/HtrA2), which act through caspase activation; and the caspase independent apoptosis-inducing factor (AIF) and endonuclease G (EndoG) (reviewed in (Kroemer et al., 2007)).

This process is strongly regulated by Bcl-2 family proteins. The anti-apoptotic members of this family have four Bcl-2 homology (Oda et al.) regions that mediate protein-protein interactions (Youle and Strasser, 2008) and contain transmembrane domains that mediate their insertion into the outer membranes of mitochondria as well as into the endoplasmic reticulum (Kelly and Strasser, 2011). This group includes Bcl-2, Bcl-w, Mcl-1, A1, BOO/DIVA, NR-13 and Bcl-x_L, which are responsible for inhibition of both MOMP and release of apoptotic factors from the mitochondria. The pro-apoptotic Bcl-2 proteins Bax, Bak, Bok, Bcl-x_S, Bcl-g_L and Bfk are multi-domain proteins with up to four BH domains, while Bid, Bad, Bik, Hrk, Puma, Noxa, Bmf and Bim have only the BH3 domain, hence called BH3-only proteins. The pro-apoptotic Bcl-2 family members reside in the cytosol but following death signaling, they are translocated to mitochondria to promote MOMP. Bad translocates to mitochondria and forms a pro-apoptotic complex with Bcl-x_L. This translocation may also be inhibited by

survival factors that induce the phosphorylation of Bad, leading to its cytosolic sequestration (Pradelli et al., 2010). Bax and Bim, in turn, translocate to mitochondria in response to death stimuli or survival factor withdrawal.

Upon release from mitochondria, cyt *c*, together with dATP and the apoptotic protease-activating factor-1 (Apaf-1), form an activation complex with caspase-9, the apoptosome. Another level of regulation is provided by the IAP family members that promote survival signaling pathways and interfere with the activation of caspases (Fulda and Vucic, 2012). Smac/DIABLO and Omi/HtrA2, in turn, can sequester and/or degrade IAPs, thereby facilitating caspase activation. AIF and EndoG do not interact with caspases and instead translocate to the nucleus to promote DNA fragmentation and chromatin condensation.

Although apparently independent, in some instances, extrinsic death signals can crosstalk with the intrinsic pathway through caspase-8-mediated proteolysis of the BH3-only protein Bid. Truncated Bid (tBid) can promote mitochondrial cyt *c* release and assembly of the apoptosome, comprising ATP, seven molecules of Apaf-1 and the same number of caspase-9 homodimers (reviewed in (Kroemer et al., 2007)).

1.1.2. p53 family of proteins

The p53 family includes the products encoded by the *p53* gene and its homologues, *p63* and *p73*. The three members of the family share a high level of similarity, which allows p63 and p73 to transactivate p53-responsive genes causing cell cycle arrest and apoptosis (Dötsch et al., 2010; Murray-Zmijewski et al., 2006). The complexity of the family has been enriched by the use of alternative promoters, splicing and translational sites (Moll and Slade, 2004; Murray-Zmijewski et al., 2006). Consequently, several protein isoforms with distinct N- and C- termini are encoded. Transcribing from P1 promoter principally gives rise to full-length isoforms with the transactivation domain (TAD), including p53, TAp63 and TAp73, whereas using the alternative P2 promoter produces N-terminal truncated isoforms without TAD, such as ΔNp53, ΔNp63 and ΔNp73 (Bourdon, 2007) (Fig. 1.1).

Combining the alternative splicing with different promoter usage, additional protein isoforms of p53, p63 and p73 arise. The p73 gene expresses at least 7 alternatively spliced C-terminal isoforms (α, β, γ, δ, ε, ζ, η) and at least 4

alternatively spliced N-terminal isoforms, which contain different parts of the transactivation domain.

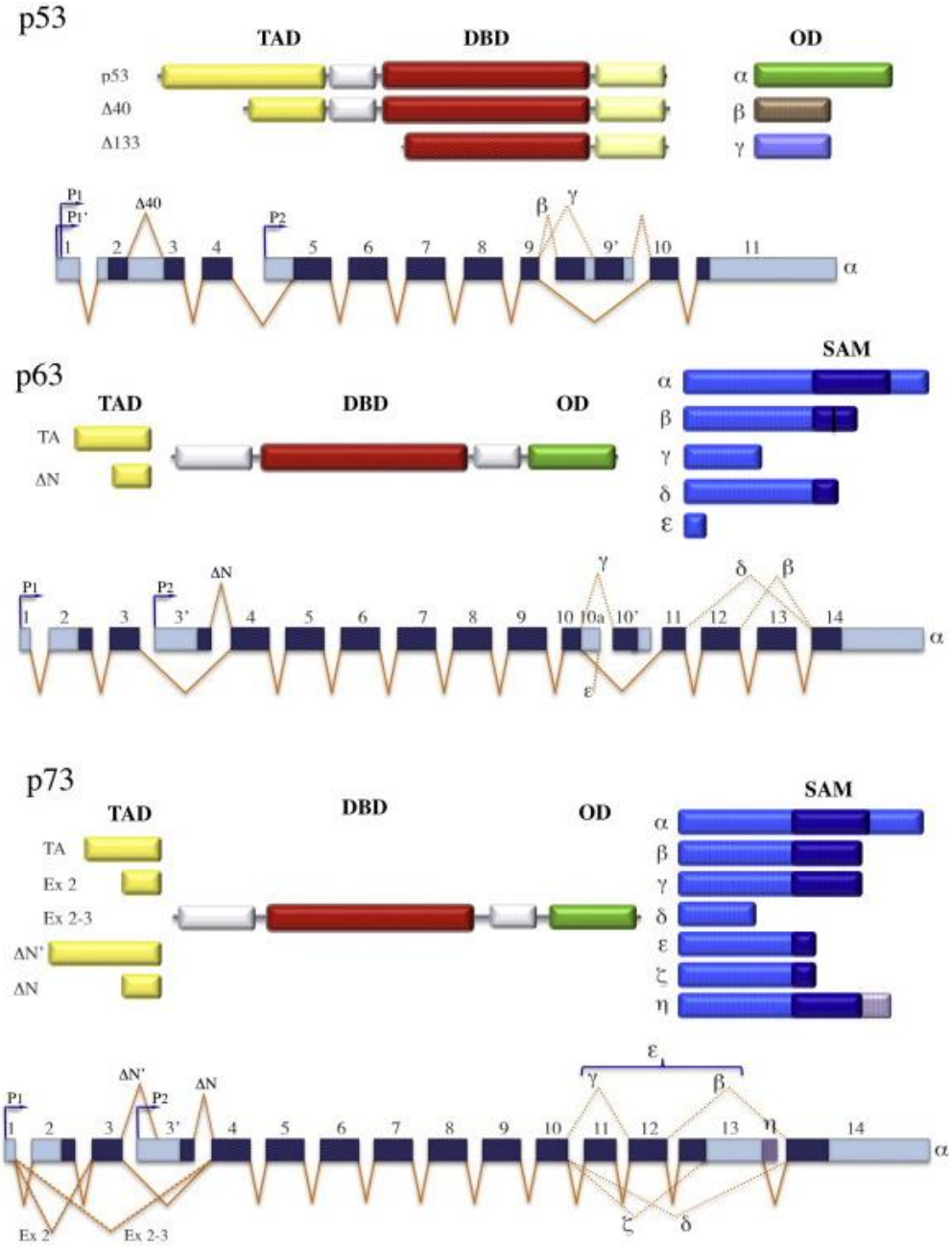


Figure 1.1. p53 family gene and protein structure. *The main p53 family protein domains include the transactivation domain (TAD), DNA binding domain (DBD), oligomerization domain (OD), and sterile alpha motif (SAM). All p53 family proteins produce two groups of mRNAs controlled by different promoters. The isoforms generated may or may not contain the transactivation domain, depending on whether they are generated by the activity of the promoter P1 (TA forms) or from the promoter P2 (ΔN forms). Adapted from Allocati et al., 2012.*

Similarly to p73, the human and mouse p63 gives rise to three different splice variants that differ in their C-termini: a full-length α form; a β form that is truncated after exon 12; and a γ form that lacks exons 12-14 and uses an additional exon 15. Each of these isoforms may or may not contain the TAD, depending on whether they are generated by the activity of the promoter upstream of exon 1 (P1) (TA forms) or from the alternative promoter in intron 3 (ΔN forms). Although ΔN isoforms of p63 do not activate transcription, they can act dominant negatively and inhibit transactivation by TAp63, TAp73 and p53 proteins, through a different transactivation domain present in their distinct N-terminal end (Helton et al., 2006).

1.1.2.1. p53

p53 functions mainly as a DNA-binding, sequence-specific transcription factor, which has well-established roles in promoting neuronal cell death during development (Tedeschi and Di Giovanni, 2009), and in adult individuals after exposure to stress and/or DNA damage. Indeed, the p53 protein is a downstream target of the DNA damage signaling network that is expressed at low levels under physiological conditions and whose activity is promoted by a wide range of stress signals; once activated, and depending on cell type and cell environment, p53 stimulates the expression of multiple pro-apoptotic genes involved in either reversible cell cycle arrest or apoptosis, including p21, GADD45, Bax, and many other, as well as targets of p63 and p73 (Irwin and Kaelin, 2001; Melino et al., 2002; Mills, 2006; Vousden and Lu, 2002).

The pro-apoptotic activity of p53 is often evident after irreversible damage in circumstances of exposure to genotoxic stress, oxidative stress or NGF withdrawal in developing or mature cortical and sympathetic neurons (Aloyz et al., 1998; Anderson and Tolkovsky, 1999; Bonaguidi et al., 2011; Cregan et al., 2004; Song and Xu, 2007). In addition, p53 regulates the expression of proteins that modulate its own activation and stability, such as the E3 ligase Mdm-2, forming multiple positive and negative feedback loops (Harris and Levine, 2005). In fact, in response to DNA damage, the p53 protein accumulates upon a number of post-translational modifications (Sims and Reinberg), which reduce its affinity for Mdm-2 and hence favor its stabilization (Kruse and Gu, 2009). Alternatively, Mdm-2-repressing mechanisms such as those mediated by the onco-suppressor

protein ADP ribosylation factor **(**ARF), which acts by sequestering Mdm-2 and preventing the Mdm-2-p53 interaction in response to oncogenic stress, can stabilize p53 (Iwakuma and Lozano, 2003).

Lethal proteins whose promoter contains a functional p53 responsive element of the activator type include pro-apoptotic members of the Bcl-2 protein family such as Bad, Bak, Bax, Bid, Bok, Noxa, and PUMA, as well as other components of the mitochondrial apoptotic pathway like the cytosolic adaptor Apaf-1, AIF, AIF-homologous mitochondria-associated inducer of death, p53-regulated apoptosis-inducing protein 1, oxidative stress-induced growth inhibitor 1, which has recently been discovered to contribute to p53-dependent MOMP in response to DNA damage, and caspase-6 (Galluzzi et al., 2011). Moreover, a number of genes are transcriptionally repressed by p53. These genes code for negative regulators of MOMP, such as Bcl-2, Bcl-x_L, and Mcl-1, as well as for anti-apoptotic proteins that operate downstream of mitochondria (Galluzzi et al., 2011).

Curiously, until recently, p53 was believed to promote MOMP only by transactivating pro-apoptotic genes and/or by repressing genes that exert anti-apoptotic effects. Recently, it has become clear that the cytoplasmic pool of p53 mediates a direct apoptogenic effect at mitochondria by physically interacting and inhibiting anti-apoptotic Bcl-2 family members (Chipuk et al., 2005; Chipuk et al., 2004; Moll et al., 2005; Morselli et al., 2008; Vaseva et al., 2009), and directly stimulating pro-apoptotic proteins, including mitochondria Bak (Pietsch et al., 2008), cytosolic Bax (Chipuk et al., 2004), Bad (Jiang et al., 2006), and Bid (Song et al., 2009). Finally, caspase-dependent proteolysis of p53 may also generate fragments that translocate to mitochondria and induce MOMP, thereby activating a feedforward loop for the amplification of the apoptotic signal (Robles et al., 2001).

1.1.2.2. p63 and p73

Much like p53, both p63 and p73 are clearly implicated in apoptosis regulation (Rocco and Ellisen, 2006). The TAp63 isoforms are pro-apoptotic, while the ΔNp63 isoforms are anti-apoptotic, similarly to the TA and ΔN isoforms of p73 (Flores et al., 2002; Wu et al., 2003).

Several studies have highlighted an essential role of endogenous TAp73 in regulating apoptosis in response to DNA damage (Jost et al., 1997; Lin et al., 2004). Upon this stress, not only ΔNp73 but also ΔNp63α (Chatterjee et al., 2008; Müller et al., 2006; Westfall et al., 2005) are rapidly degraded. Thus, they do not exert their dominant negative effect on p53, TAp63 and TAp73, allowing cell cycle arrest and apoptosis to proceed. Moreover, previous studies have already shown that treatment of cells with various DNA-damaging agents resulted in increased expression of TAp63 and TAp73 isoforms (Hershkovitz Rokah et al., 2010; Sayan et al., 2007b; Yao et al., 2010c).

p73 is a target of the tyrosine kinase c-Abl in response to DNA damage induced by cisplatin or ionizing radiation, thereby resulting in apoptosis (Agami and Bernards, 2000; Gong et al., 1999; Yuan et al., 1999). In turn, the role of p63 in apoptosis pathways is still under debated. Gressner *et al*. (Gressner et al., 2005) described the role of TAp63α in activating apoptosis through death receptor signaling and mitochondria by activating transcription of p53 targets (Testoni et al., 2006). In fact, it was shown that TAp63 can induce the expression of death receptors including Fas, TNF-R, and TRAIL-R, as well as pro-apoptotic Bcl-2 family members, such as Bax (Gressner et al., 2005). Furthermore, inhibition of TAp63 function results in chemoresistance. Sayan *et al.* (Sayan et al., 2007b) have also demonstrated that after induction of apoptosis, the transactivation-inhibitory domain of the ΔNp63α isoforms is cleaved by activated caspases. Cleavage of ΔNp63α relieves its inhibitory effect on transcriptionally active p63 proteins, and the cleavage of TAp63α results in production of a TAp63 protein with enhanced transcriptional activity, promoting apoptosis.

Nevertheless, the involvement of p63 and p73 in neural p53-mediated apoptosis is still controversial (Benchimol, 2004). In fact, there is intense debate on whether and how p63 and p73 interact with p53 in apoptosis context. During development, the p53 family has been considered pivotal in determining the life *versus* death of developing peripheral sympathetic neurons (Jacobs et al., 2005; Pozniak et al., 2000; Walsh et al., 2004). p53 has a partial but essential role for efficient neuronal apoptosis *in vivo* (Aloyz et al., 1998; Jacobs et al., 2005; Slack et al., 1996). TAp63 can induce neuronal apoptosis on its own, but p53 requires TAp63, indicating that p63 is dominant to p53 during developmental sympathetic neuronal death (Jacobs et al., 2006). In addition, analysis of the embryonic

superior cervical ganglion shows that TAp63 is also essential for naturally occurring sympathetic neuronal death *in vivo*. Sympathetic neuron survival is largely regulated by pro-apoptotic signals deriving from $p75^{NTR}$ and pro-survival signals deriving from the NGF/TrkA receptor (Kaplan and Miller, 2000). In addition, NGF withdrawal and $p75^{NTR}$ activation converge on TAp63 and p53, which then activate Bax gene and protein expression to induce mitochondrial apoptosis (Jacobs et al., 2005). Interestingly, Dugani *et al.* (Dugani et al., 2009) have also demonstrated that the ΔNp63 isoform is crucial for the survival of neural precursors, namely embryonic cortical precursors, and newly born neurons, antagonizing the pro-apoptotic actions of p53. NGF induced anti-apoptotic ΔNp73 protein functions, at least in part, by antagonizing the pro-apoptotic actions of p53 (Pozniak et al., 2000), and potentially TAp63. Thus, p73 acts as an essential pro-survival protein (Lee et al., 2004; Pozniak et al., 2000). Thus, coordinated regulation of the abundance of p53 family proteins appears to be critical in influencing the outcome of neural apoptosis.

1.1.3. JNK/c-Jun signaling pathway

Several stimuli including growth factors, proinflammatory cytokines, neurotransmitters, cell-matrix interactions, physical and chemical stresses activate MAPK signaling pathways (Chang and Karin, 2001; Ip and Davis, 1998; Karin et al., 1997; Whitmarsh and Davis, 1996). In mammalian cells, the MAPK family is comprised of 3 groups: c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase, and p38. The JNK pathway is a central stress signaling pathway implicated in neuronal plasticity, regeneration and death (Herdegen et al., 1997). Signaling cascades activate the activator protein-1 (AP-1) through the phosphorylation of distinct substrates. AP-1 is linked to various cellular responses, including proliferation, differentiation, oncogenic transformation and apoptosis (Angel and Karin, 1991; Shaulian and Karin, 2002). The AP-1 capacity to control several biological processes is primarily due to its structural and regulatory complexity. In fact, AP-1 is a collective term referring to dimeric basic/leucine zipper transcription factors that belong to several subfamilies, including the Jun (Estus et al., 1997) subfamily. These DNA binding proteins may form homodimers or heterodimers with each other (Chinenov and Kerppola, 2001). c-Jun is an essential regulator of both cell survival and death, with its levels being increased upon exposure to a variety of stress

signals and chemotherapeutic agents as well as growth factors (Eferl and Wagner, 2003). Its activity is regulated by phosphorylation at serines 63 and/or 73 through JNKs (Dérijard et al., 1994; Ham et al., 1995; Kyriakis et al., 1994).

The detailed mechanisms by which c-Jun regulates apoptosis is not fully understood. The role of c-jun in apoptosis may be stimulus- and cell type-specific. JNK increases the ability of c-Jun to activate the transcription of target genes (Estus et al., 1994; Ham et al., 1995) that are more proximal to promote caspase activation and neuron apoptosis (Edwards and Tolkovsky, 1994; Putcha et al., 2000). Indeed, induction of pro-apoptotic Bcl-2 family members Bim (Whitfield et al., 2001) and death protein 5/harakiri were shown to be JNK/c-Jun-dependent in neurons (Harris and Johnson, 2001). In addition, some studies have reported that after NGF withdrawal in sympathetic neurons, c-Jun protein levels increased and the N-terminal c-Jun transcriptional activation domain became more phosphorylated (Eilers et al., 1998; Eilers et al., 2001), in correlation with an increase in JNK activity (Bruckner et al., 2001; Eilers et al., 1998). Moreover, microinjection of an expression vector for a c-Jun dominant negative mutant, which inhibited AP-1 activity, resulted in protection of neurons against NGF withdrawal-induced death, whereas overexpression of wild-type c-Jun was sufficient to induce apoptosis in the presence of NGF (Estus et al., 1994; Ham et al., 1995). The role for c-Jun in CNS neurons has also been reported, namely in cerebellar granule neurons (Watson et al., 1998). In addition, the c-Jun pathway has also been implicated in the induction of apoptosis in differentiated PC12 cells deprived of NGF (Xia et al., 1995), in striatal neurons treated with neurotoxic concentrations of dopamine (Luo et al., 1998), or in a hippocampal neuron cell line transfected with an expression vector for polyglutamine-expanded Huntingtin (Liu, 1998).

Curiously, JNK may also phosphorylate p53 (Fuchs et al., 1998), and subsequently up-regulate Bax (Miyashita and Reed, 1995). However, the interplay between p53 and c-Jun in regulating neuronal survival and death genes remains to be determined. Regarding p73, it was already demonstrated that JNK and c-Jun is required for the stabilization of TAp73 (Jones et al., 2007; Toh et al., 2004) and degradation of ΔNp73 under stress stimulation (Dulloo et al., 2010). Importantly, c-Jun has also been shown to regulate the abundance of p63. Yao *et al.* (Yao et al., 2010b) showed that the transcriptional activity of the TAp63 promoter and TAp63

protein levels were both up-regulated by an increased c-Jun activity in the Hep3B human hepatocellular carcinoma cell line.

However, since c-Jun null mice die during embryonic development at embryonic day 12.5 (E12.5) due to hepatic failure (Hilberg et al., 1993), *in vivo* studies focused on the role of c-Jun in the nervous system were only possible due to knockout (KO) mice models for the three mammalian JNK genes, J*nk1*, *Jnk2*, and *Jnk3*. JNK3 is mainly expressed in the CNS (Junyent et al., 2011), whereas JNK1 and JNK2 are expressed in many other tissues (Ip and Davis, 1998). In fact, c-Jun phosphorylation and neuronal cell death was inhibited in neurons from JNK3 null mice (Bruckner et al., 2001). Finally, JNK1/JNK2 double KO mice, but not single, showed that both JNKs are required for developmental cell death in the neural tube, whereas in the developing cortex they promote neuronal survival (Kuan et al., 1999; Sabapathy et al., 1999). Thus, although JNKs and c-Jun may be pro-apoptotic in many neuronal cell types, they can have other cellular roles.

1.1.4. Role of apoptosis in Alzheimer's disease

AD is the most common human age-related, sporadic neurodegenerative disorder, characterized by a global, progressive and irreversible cognitive decline that is strongly correlated with synaptic dysfunction and death of neurons from hippocampus and associated regions of the cerebral cortex. The affected brain shows extracellular deposits of Aβ peptides, known as amyloid or senile plaques (Mattson, 2004), and intracellular accumulation of paired helical filaments that form NFTs (McKee et al., 1990), consisting of polymerized hyperphosphorylated tau protein (Spillantini and Goedert, 1998). Although activation of apoptotic signaling has been reported in AD brains (Raynaud and Marcilhac, 2006), the precise intracellular signaling pathways by which Aβ peptides trigger cell death are not fully understood.

The study of apoptosis in neurodegenerative diseases has become a challenging task and a controversial area of research, especially because synaptic loss and electrophysiological abnormalities typically precede cell loss. In addition, given the slow progression of most neurodegenerative diseases, in contrast with the rapid progression of a cell through apoptosis, the detection of representative apoptotic neurons in AD patients could be difficult. Further, apoptotic processes may be terminated or not yet initiated by the time of tissue examination. In fact, the collected

tissue is usually representative of the end-stage disease, being removed much later than it would be ideal to treat patients and prevent neuronal cell death. Finally, the criteria used to classify the type of cell death as apoptosis has often been based on morphological assessment and biochemical assays, which may also account for other forms of programmed cell death.

Although many AD mouse models have been reported, there is no single model that exactly mimics human AD (Elder et al., 2010). Thus, it is important to keep in mind the limitations when research data generated from using these AD animal models are interpreted.

Knowledge of the production and degradation pathways acting on the toxic proteins that cause AD, and various other proteinopathies, could help in the design and improvement of therapeutic strategies. Specifically, the reduction of Aβ load has given many insights into drug development strategies for potential therapies in AD aimed at preventing Aβ formation or accelerating its degradation. β- and γ-secretases have been considered as possible drug targets. Targeting secretases may, however, have unforeseen effects because a physiological role for Aβ has also been proposed (Atwood et al., 2003; Chen and Dong, 2009; Pearson and Peers, 2006).

1.1.4.1. Aβ production and accumulation

The amyloid precursor protein (APP) in mammals belongs to a family of conserved type I membrane proteins that include APP, APP-like protein 1 (Aydin et al.) and 2 (APLP2) (Aydin et al., 2012). Although the APP family is abundantly expressed in the brain and APLP1 expression is restricted to neurons (Lorent et al., 1995), APP and APLP2 can also be found in the majority of other tissues. The human *APP* gene encodes a single transmembrane polypeptide, whose primary structure contains a signal peptide for secretion, a large extracellular N-terminal domain, a transmembrane domain, and a cytoplasmic C-terminal tail.

During normal development, APP can undergo amyloidogenic or non-amyloidogenic processing via cleavage by three different secretases, α-, β-, and y-secretases (Ling et al., 2003). In the non-amyloidogenic pathway, cleavage of APP occurs via presenilin-containing α-secretase complex, which cleaves within Aβ sequence and consequently abrogates Aβ formation, leading to the release of a large soluble extracellular fragment (Mattson), which can be neuroprotective, and retention of an 83-residue membrane-bound fragment (C83). Alternatively, APP can be

cleaved by β-secretase, which cuts at the N-terminus of Aβ, generating a smaller extracellular fragment (Mattson) and a 99-residue membrane-bound fragment (C99). Peptide fragments C83 and C99 may then undergo conformational alterations and become targets for the presenilin (PS1/PS2)-dependent γ-secretase, which cleaves within the plasma membrane domain. C99 proteolysis generates $A\beta_{1-40}$ and $A\beta_{1-42}$ (Selkoe and Wolfe, 2007). The cleavage of either C83 or C99 by γ-secretase may also generate an amyloid intracellular domain (AICD), which moves to the nucleus where it may modulate transcription of target genes.

Aβ is produced as a monomer, but readily aggregates to form multimeric complexes. Therefore, the term "soluble Aβ" is generally applied either to newly generated, cell-secreted Aβ or to that fraction of tissue or synthetic Aβ that is taken into the aqueous phase of a non-detergent containing extraction buffer. In contrast, "misfolded" and "aggregated" Aβ are terms used to describe very early, nonspecific changes in Aβ folding states or solubility states, respectively. In addition, "oligomeric" Aβ refers to peptide assemblies with limited stoichiometry, such as dimmers and trimers, while protofibrils are structures of intermediate order between aggregates and fibrils (Gandy, 2005).

Aβ peptide is detected in human cerebrospinal fluid (CSF) as a range of isoforms between 38 and 43 amino acids in length. The predominant isoforms secreted are $A\beta_{1-40}$ (90%) whereas $A\beta_{1-42}$ isoform represents less than 10% (Thinakaran and Koo, 2008). In patients with AD, the relative proportions of $A\beta_{1-40}$ and $A\beta_{1-42}$ change to approximately 50% each (Mehta et al., 2001). The accumulation of Aβ is progressive and strongly accepted as an important contributor to the neuronal dysfunction and loss (Hardy and Selkoe, 2002). The pattern and distribution of the different Aβ species varies among the different topographical lesions.

1.1.4.2. Cytotoxicity of Aβ

Several studies suggest that Aβ-induced oxidative stress leads to apoptotic neuronal cell death that can be inhibited by antioxidants (Behl et al., 1994; Mattson and Goodman, 1995; Pillot et al., 1999). In fact, many lines of evidence suggest that mitochondria dysfunction and oxidative damage have a central role in aging-related neurodegenerative diseases, being key regulators of cell survival and death (Danial and Korsmeyer, 2004).

The involvement of apoptosis in AD has been corroborated by studies showing that Aβ alters the expression of the Bcl-2 family of apoptosis-related genes (Yao et al., 2005) and that survival pathways play a pivotal role in preventing Aβ-mediated apoptosis (Watson and Fan, 2005). On the other hand, studies in an anti-NGF transgenic mouse model and PC12 cells suggest that discontinued or limited NGF supply induces an overproduction of Aβ peptides, triggering downstream apoptotic cell death (Capsoni et al., 2000; Matrone et al., 2008). Furthermore, $p75^{NTR}$ has been consistently linked to changes occurring in AD (Schliebs, 2005; Sotthibundhu et al., 2009), including cell death of basal forebrain cholinergic neurons, where this receptor is highly expressed (Dechant and Barde, 2002). Both *in vitro* and *in vivo* evidence suggests that the JNK/c-Jun cascade is a critical event during neuronal death in AD (Anderson et al., 1994; Kihiko et al., 1999; Viana et al., 2010), where JNK mediates the activation of several molecules, including caspase-2 (Viana et al., 2010), p53 (She et al., 2002), and Bcl-2 family members (Sorenson, 2004) and potentiate inflammatory responses via AP-1 activation (Manning and Davis, 2003). Viana *et al.* (Viana et al., 2010) showed that Aβ exposure in PC12 cells resulted in activation and nuclear translocation of JNK, and caspase-2 activation. Caspase-2 triggers apoptosis through activation of the mitochondrial apoptosis pathway. The induction of c-Jun was shown to occur *in vitro* after Aβ treatment (Estus et al., 1997; Iwasaki et al., 1996; Kihiko et al., 1999) and in AD brains (Anderson et al., 1994; Anderson et al., 1996), suggesting a possible requirement for this transcription factor in Aβ toxicity.

Curiously, p53 family members have also been associated with Aβ-induced neuronal apoptosis. The first indication that the p53 family may play a role in AD came in 1996 with the demonstration that intracellular Aβ up-regulates p53 in the brains of transgenic mice overexpressing the Aβ fragment of APP (LaFerla et al., 1996). The use of pifithrin-α, an inhibitor of p53-dependent gene transcription, was shown to protect neurons against Aβ-induced apoptosis (Culmsee et al., 2001; Hooper et al., 2007). p53 was also found to be up-regulated in the brain of AD patients [43, 44]. In 2004, Caricasole *et al.* (Caricasole et al., 2004) showed that Aβ activates the expression of the soluble Wnt antagonist Dickkopf-1 (Caricasole et al.), a p53 target gene. Knockdown of Dkk1 in primary neurons, in turn, almost completely blocked Aβ-induced tau phosphorylation, implicating the p53 family in the "amyloid cascade" pathway.

More recently, it has been demonstrated that p53 participates in Aβ-induced apoptosis of PC12 neuronal cells through modulation of Bax expression (Ramalho et al., 2004). Furthermore, Aβ-induced apoptosis in rat primary cortical neurons was associated with translocation of Bax to the mitochondria, followed by cyt *c* release, caspase activation, and DNA and nuclear fragmentation (Solá et al., 2003). Interestingly, a novel biological function of intracellular $A\beta_{1-42}$ has been suggested, where it acts as a transcription factor for the p53 promoter, enhancing p53-dependent neuronal apoptosis in AD (Ohyagi et al., 2005).

Several reports have also demonstrated p73 involvement in apoptosis triggered by $A\beta_{25-35}$, being probably vital for mediating the process of AD (Zhang et al., 2011). It has been shown that in primary cortical neurons generated from p73 KO mice, the activity of JNK increased, while JNK inhibition decreased tau phosphorylation in these neurons (Wetzel et al., 2008). Since ΔNp73, and not TAp73, can bind and inhibit JNK, the loss of ΔNp73 forms may result in tau phosphorylation and neurodegeneration. As mentioned before, Aβ peptide activates c-Abl and increases p73 levels (Alvarez et al., 2004; Vázquez et al., 2009), while the Swedish mutant form of APP increases expression from both the TA and ΔN promoters of the p73 gene, only inducing an overall increase in the TA forms (Vázquez et al., 2009). Surprisingly, the mechanisms regulating p63 levels under Aβ-mediated apoptosis have yet to be elucidated.

Aβ peptides accumulate within mitochondria, strongly affecting their function and morphology, particularly in the synaptic compartment (Balietti et al., 2012). Increasing evidence indicates that the mitochondrial dysfunction is an important early factor in the development of AD-like pathology (Blass and Gibson, 2006; Yao et al., 2009). Accumulation of Aβ in mitochondria appears to be also associated with diminished enzymatic activity of respiratory chain complexes III and IV, and reduction of oxygen consumption (Caspersen et al., 2005).

Finally, several studies have demonstrated that Aβ triggers the formation of tau pathology. Inflammatory processes are strongly correlated with AD onset and progression in humans, and could have a pivotal role in disease etiology (Kitazawa et al., 2005; Krstic and Knuesel, 2013). Interestingly, it has been suggested that different forms of Aβ may be responsible for inducing tau hyperphosphorylation in different settings (Blurton-Jones and Laferla, 2006; LaFerla, 2010).

1.1.5. Regulation of apoptosis by autophagy

Autophagy is a cellular process in which isolated membrane sequesters part of the cytoplasm to form a double-membrane vesicle, called autophagosome (AP) that fuses with lysosomes for the degradation of its contents, long-lived cytoplasmic proteins or damaged organelles, by acidic lysosomal hydrolases, maintaining normal cell homeostasis (Nixon and Yang, 2011). There are at least three types of autophagy; macroautophagy (delivery of cytosolic contents to the lysosome by autophagosomes), microautophagy (inward invagination of the lysosomal membrane) and chaperone-mediated autophagy (direct translocation across the lysosomal membrane). Among these, macroautophagy referred to as autophagy has been the most studied. Autophagy-related (Atg) proteins are a set of evolutionarily conserved gene products, originally identified in yeast and followed by the identification of homologs in higher eukaryotes (Huang and Klionsky, 2002), which are required for autophagy.

Among Atg proteins, one subset is essential for AP formation, and is referred to as the "core" molecular machinery (Xie and Klionsky, 2007). In mammalian cells, most of the Atg proteins are observed in isolated membranes but not in complete APs (Longatti and Tooze, 2009; Tooze and Razi, 2009). Only microtubule-associated protein light chain 3 (LC3), a mammalian homolog of yeast Atg8, is known to exist in APs, and therefore, this protein serves as marker for APs and is widely used to monitor autophagy (Kabeya et al., 2000).

Since autophagy can block apoptosis, one might expect that their regulation would be tightly coordinated. Curiously, the same proteins can regulate both processes. Some molecular connections occur upstream of the autophagic and apoptotic machinery itself, where signaling pathways regulate both processes (Thorburn, 2008). p53, which is a potent inducer of apoptosis, can also induce autophagy through increased expression of a direct p53 target gene called DRAM (Crighton et al., 2006). Similarly, activation of the phosphatidylinositide 3 kinase (PI3K)/Akt pathway, which is well-known to inhibit apoptosis, also inhibits autophagy (Arico et al., 2001). Thus, important signaling pathways simultaneously increase or decrease both autophagy and apoptosis. In addition, proteins that are themselves central components of the apoptosis machinery, including Bcl-2 family proteins and FADD regulate both processes directly. In addition, in normal cytoprotective autophagy, activation of JNK can up-regulate Beclin-1 (Li et al.,

2009), a marker for autophagy activation that was originally identified as an interaction partner of the anti-apoptotic protein Bcl-2 (Kang et al., 2011). When Bcl-2 and Bcl-x_L proteins form Beclin-1-Bcl-2/Bcl-x_L complexes, the pro-autophagic function of Beclin-1 is inhibited (Maiuri et al., 2007).

Although autophagy is crucial in survival and suppression of apoptosis, it can also lead to cell death (Lum et al., 2005; Mizushima, 2005; Yu et al., 2004b), either in collaboration with apoptosis or as a backup mechanism when apoptotic machinery is disabled (Eisenberg-Lerner et al., 2009; Shimizu et al., 2004; Yu et al., 2004a). One example of autophagic cell death is caused by increased ROS resulting from autophagic degradation of catalase (Yu et al., 2006). Interestingly, neurons presenting accumulation of autophagic vacuoles (AVs) seem to have dysfunction of this degradation pathway (Nixon et al., 2005; Yu et al., 2005; Yu et al., 2004c). Autophagy is normally efficient in the brain as reflected by the low number of AVs at any given moment (Boland et al., 2008). Interestingly, it has been reported an elevated number of AVs in brains of AD patients and in transgenic mouse models, associated with dystrophic neuritis and deformed synaptic membranes (Nixon et al., 2005; Yu et al., 2005). This suggests impairment of clearance mechanisms, thus indicating that autophagy is involved in AD pathogenesis. In addition, Lipinski *et al.* (Lipinski et al., 2010) has shown that ROS play an essential role in mediating autophagy upstream of PI3K in brains of AD patients. This pathway is critical for the initiation of autophagy by Aβ. Zheng *et al.* (Zheng et al., 2006b) have also demonstrated that ROS induce intralysosomal Aβ accumulation in cultured differentiated neuroblastoma cells through the activation of autophagy. However, it remains to be elucidated whether intralysosomal Aβ corresponds to autophagocytosed cytoplasmic Aβ, or a derivative of autophagocytosed APP, or both.

Interestingly, autophagy has also been reported as a mechanism of neuronal protection from Aβ-induced cytotoxicity (Cheung et al., 2011; Hung et al., 2009; Wang et al., 2010), and serves as a mechanism that mediates clearance of ubiquitinated Aβ to eliminate molecular debris and restore neurotransmitter balance (Khandelwal et al., 2011) (Fig. 1.2). In fact, Hung *et al*. (Hung et al., 2009) have demonstrated in SH-SY5Y/pEGFP-LC3 cells that Aβ treatment activates autophagy, and inhibition of this process increases neuronal death, indicating that the clearance of Aβ plays a neuroprotective role. Furthermore, the decreased activity of the proteasomal system and consequent accumulation of Aβ induced autophagy (Iwata et

al., 2005; Khandelwal et al., 2011), concurrent with ubiquitination (Nedelsky et al., 2008; Ravikumar and Rubinsztein, 2004; Wong and Cuervo, 2010). When proteins are aggregate-prone and poor proteasome substrates, autophagy becomes a major clearance route by default.

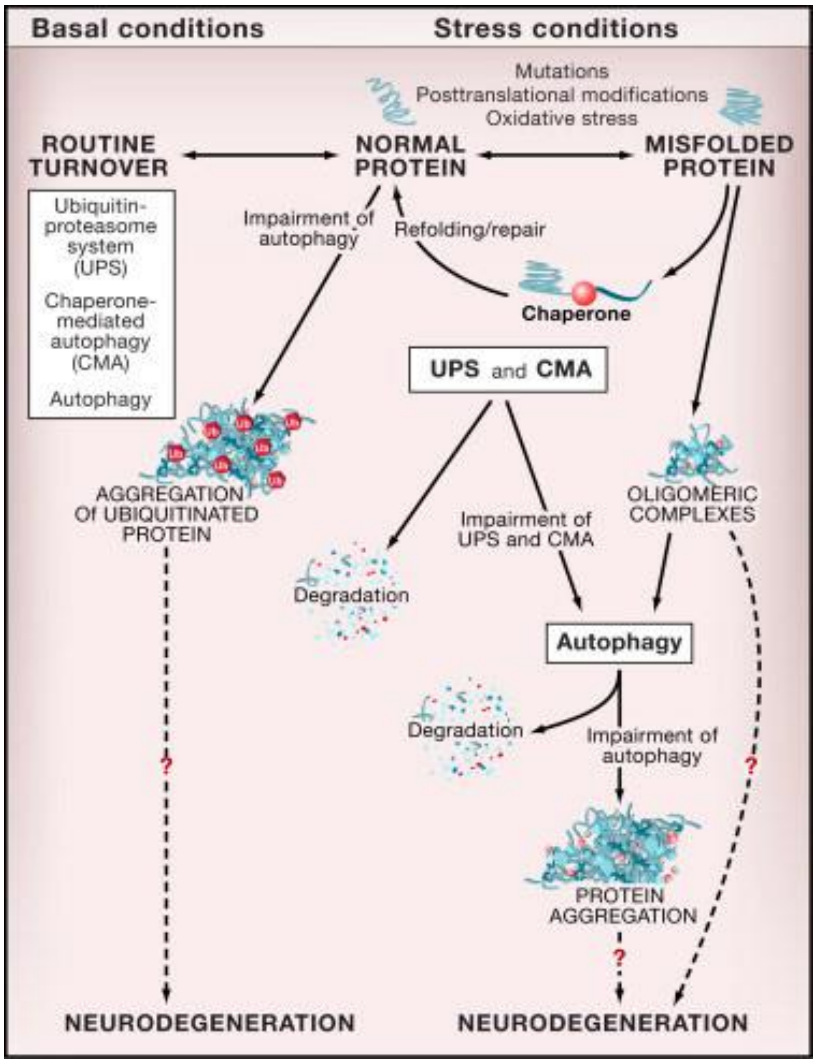


Figure 1.2. Autophagy is crucial for intracellular clearance of neurons. *Normal proteins are routinely turned over by different protein degradation systems, including the ubiquitin-proteasome system (UPS), chaperone-mediated autophagy (CMA), and autophagy. In autophagy-deficient neurons, there is an accumulation of ubiquitinated protein aggregates that is associated with neurodegeneration. Proteins altered by mutations, posttranslational modifications, or stress undergo a conformational change, are recognized by molecular chaperones, and are either refolded and repaired or delivered to protein degradation systems, usually UPS or CMA. If these protein degradation systems are impaired or if the altered proteins form oligomeric complexes that cannot be recognized by the UPS or CMA, autophagy may be the primary route for the removal of these abnormal and potentially toxic proteins. Impaired autophagy is associated with the formation of protein aggregates and increased neurodegeneration. Adapted from Levine and Kroemer, 2008.*

In 3xTg-AD mice, the E3-ubiquitin ligase parkin clears vesicle-containing defective mitochondria, suggesting that the clearance of intraneural Aβ reduces oxidative stress (Geisler et al., 2010; Khandelwal et al., 2011), and may modulate autophagy. These issues deserve further investigation since autophagy might be one of the key checkpoints responsible for neural differentiation, as an alternative to cell death.

1.1.6. Modulation of neural apoptosis by bile acids

Bile acids are the major component of bile, produced in the liver and secreted via the bile ducts and gallbladder into the intestine (Setchell et al., 1992), where they act as detergents being crucial in the solubilization of lipids in the human intestinal lumen. Ursodeoxycholic acid (UDCA) is an endogenous bile acid used during the past several decades for the treatment of certain liver diseases (Lazaridis et al., 2001; Paumgartner and Beuers, 2002). It is normally present in human bile in low concentrations. The anti-apoptotic effects of UDCA have been demonstrated both *in vivo* and *in vitro* in the rat liver [48, 68], and in human hepatocytes [69]. Furthermore, it has been shown that both UDCA and its taurine conjugated form, tauroursodeoxycholic acid (TUDCA), play a unique role in modulating cell death in response to a variety of agents, by interrupting classic pathways of apoptosis in both hepatic and non-hepatic cells, including neurons (Rodrigues et al., 1998; Rodrigues et al., 2002a; Rodrigues et al., 2000b; Solá et al., 2003) (Fig. 1.3**).** A possible explanation for this ubiquitous anti-apoptotic effect appears to involve the mitochondrial membrane stabilization (Rodrigues et al., 2003b). Indeed, both UDCA and TUDCA are antioxidant molecules (Oveson et al., 2011; Phillips et al., 2008), modulating mitochondrial apoptosis in a number of animal models (Rodrigues et al., 1998; Rodrigues et al., 2002a; Rodrigues et al., 2000b). In fact, UDCA and, more efficiently, TUDCA inhibited bilirubin- and Aβ-induced MOMP and consequent cyt *c* release in isolated mitochondria of neural and glial cells (Rodrigues et al., 2000a). Moreover, using electron paramagnetic resonance spectroscopy analysis, it has been demonstrated that TUDCA prevents Aβ-driven modifications in mitochondrial membrane redox status, lipid polarity and protein order (Rodrigues et al., 2001). The effects of TUDCA on the Aβ-affected mitochondria were further confirmed by the

observation of decreased Bax translocation in the presence of this bile acid (Solá et al., 2003).

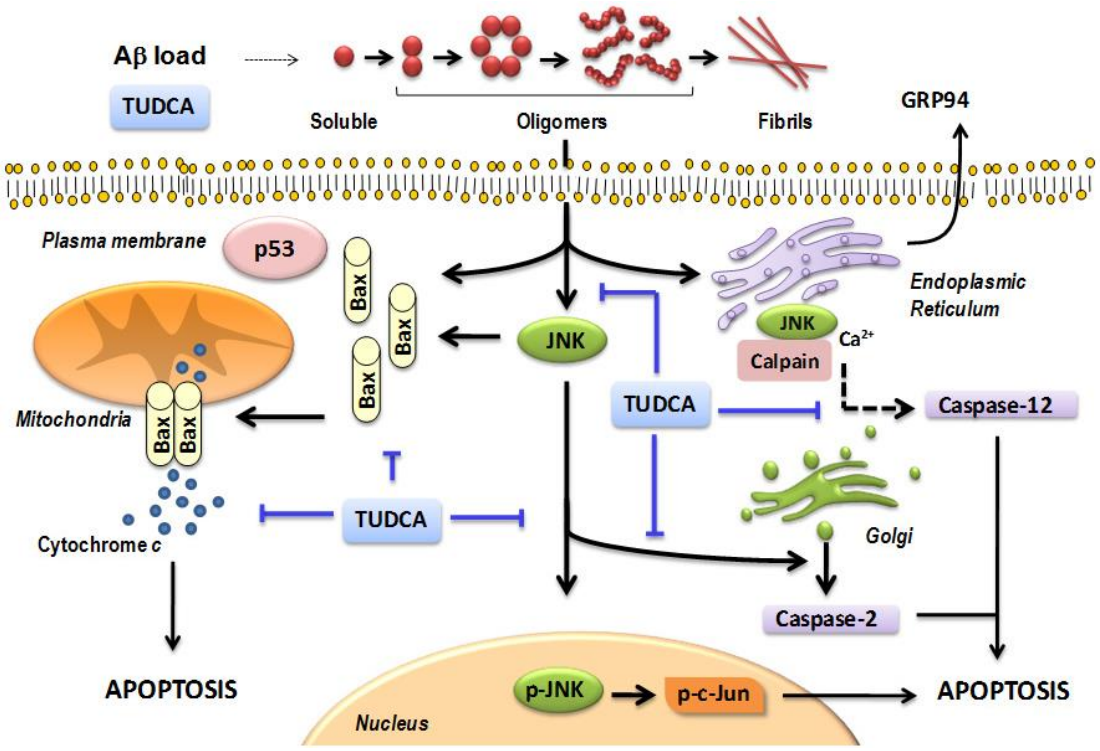


Figure 1.3. Tauroursodeoxycholic acid modulates amyloid ß-induced toxicity by inhibiting organelle-driven apoptosis. *Soluble Aβ is a hydrophobic peptide that is prone to aggregation. Equilibrium is produced between several extracellular and intracellular Aβ species, including monomeric, oligomeric, and fibrillar forms. Cell death by toxic Aβ appears to involve a complex signaling network of inter-organellar cross-talk that involves mitochondria, ER, Golgi and nucleus. TUDCA effectively abrogates this signaling network. Intracellular calcium (Ca^{2+}) free levels are tightly regulated by the ER to avoid cell death induced by Ca^{2+} deregulation. Inside the ER, Ca^{2+} is maintained elevated by Ca^{2+}-binding buffering proteins, such as GRP94, promoting cellular Ca^{2+} homoeostasis. Caspase-12 can be activated via Ca^{2+}-activated protease calpain during ER stress-induced apoptosis. Aβ-induced cell death requires the activation of caspase-2, which is localized at the Golgi complex and has been described as a target of the JNK pathway that triggers apoptosis through activation of the mitochondrial pathway. In fact, Aβ, p53 and activated JNK trigger Bax translocation to the mitochondria, formation of pore complex, cytochrome c release, and subsequent mitochondrial apoptosis. In addition, JNK activates the pro-apoptotic transcription factor c-Jun, enhancing the expression of apoptosis proteins. Adapted from Viana et al., 2012.*

In addition, UDCA and TUDCA interfere with upstream targets of mitochondria, in a caspase-independent manner (Solá et al., 2003b). The anti-apoptotic effects of these bile acids appear to be dependent on other molecular

targets upstream of the E2F-1/p53 apoptotic pathway (Solá et al., 2003b), namely the nuclear steroid receptors. In fact, as cholesterol-derived molecules, UDCA and TUDCA interact with specific regions of glucocorticoid (GR) and the mineralocorticoid (MR) receptors (Solá et al., 2006; Solá et al., 2005; Solá et al., 2004). Aβ incubation reduces MR nuclear translocation while increasing nuclear GR levels. Notably, pretreatment with TUDCA markedly altered Aβ-induced changes in nuclear steroid receptors.

The protective role of TUDCA has been extended to several mouse models of neurological disorders, including Huntington's disease (Keene et al., 2002), Parkinson's disease (Duan et al., 2002), and acute ischemic (Rodrigues et al., 2002b) and hemorrhagic stroke (Rodrigues et al., 2003a). Notably, in an *in vitro* AD pathologic model, TUDCA prevented the reduction of dendritic spine number and the decrease in the frequency of spontaneous excitatory synaptic activity (Ramalho et al., 2013; Shankar et al., 2007), supporting the idea that the protective role of this bile acid goes beyond its capacity to modulate neuronal death.

It is becoming increasingly evident that activation of survival pathways may also represent an important additional mechanism by which TUDCA inhibits apoptosis. In primary rat hepatocytes, TUDCA was shown to protect mitochondria-controlled apoptosis by activating the PI3K and the MAPK survival pathways (Bradham et al., 1998; Schoemaker et al., 2004). PI3K signaling is also activated by TUDCA in Aβ-induced neuronal apoptosis (Solá et al., 2003). In fact, incubation with wortmannin, an inhibitor of the PI3K pathway, significantly reduced the ability of TUDCA to modulate p53-induced apoptosis (Ramalho et al., 2006). Moreover, by interfering with apoptotic pathways, TUDCA not only increases the survival of neurons but also prevents downstream abnormal conformations of tau (Ramalho et al., 2008). Finally, TUDCA was shown to abrogate Aβ-induced apoptosis of neuronal PC12 cells by inhibiting the E2F-1/p53 apoptotic pathway, hence modulating the expression of Bcl-2 family members (Ramalho et al., 2004). Similar results were seen *in vitro* models of familial AD that consist of mouse neuroblastoma cells expressing APP with the Swedish mutation or double-mutated human APP and PS1 (Ramalho et al., 2006), as well as in primary rat neurons incubated with Aβ (Solá et al., 2003). A recent study in primary rat cortical and hippocampal cultures exposed to Aβ, and brain tissue of APP/PS1

transgenic mice also showed that TUDCA suppresses Aβ-induced decrease of the neuronal marker postsynaptic density-95 protein (PSD-95). Of note, TUDCA was capable of reducing Aβ deposits, glial activation (Itagaki et al., 1989) and neuronal integrity loss in the APP/PS1 double transgenic mouse model of AD, rescuing memory and learning deficits (Nunes et al., 2012). This effect may be partially explained by the effect of TUDCA in regulating lipid-metabolism mediators that influence Aβ production and intracellular accumulation (Nunes et al., 2012).

1.2. Neural Differentiation

The defining hallmark of metazoan multicell life forms is cell specialization and adaptation that results from the process commonly referred to as differentiation. In fact, during differentiation, stem cells give rise to committed and specialized cells. Stem cells are unspecialized precursor cells, capable of generating identical progeny (self-renewal), thereby sustaining a stem cell pool, with the ability to develop into other cell types (differentiation) carrying out particular tissue functions. *In vivo*, stem cells are thought to reside in specific cellular niches that constitute privileged sites for support of self-renewal (Doetsch, 2003; Spradling et al., 2001; Watt and Hogan, 2000). They can be found both in the embryo and adult organisms, and may also differ in their differentiation potential. Stem cells can be somatic (fetal or adult) or embryonic. Embryonic stem cells (ECSs) have attracted a lot of attention because of their origin and plasticity, being currently used to create transgenic animals, and proposed for use in a wide variety of commercial and clinical applications (Gage, 2000; Thomson et al., 1998). These cells are derived from the inner mass cell of developing blastocysts (Evans and Kaufman, 1981; Martin, 1981) and are considered pluripotent, capable of transforming into cells from all three somatic germ layers as well into germ cells. In contrast, adult stem cells are considered multipotent because they only originate different cell types of a particular lineage.

Neural stem cells (NSC) are defined as clonogenic cells, having some capacity of self-renewal, and also as multipotent cells, being able to differentiate into neuronal and glial precursor cells (Brüstle et al., 1997) (Fig. 1.4). Neuronal precursor cells (NPCs) can further generate motor, sensory, or dopaminergic

neurons, while glial precursor cells generate astrocytes or oligodendrocytes (Reynolds and Weiss, 1996).

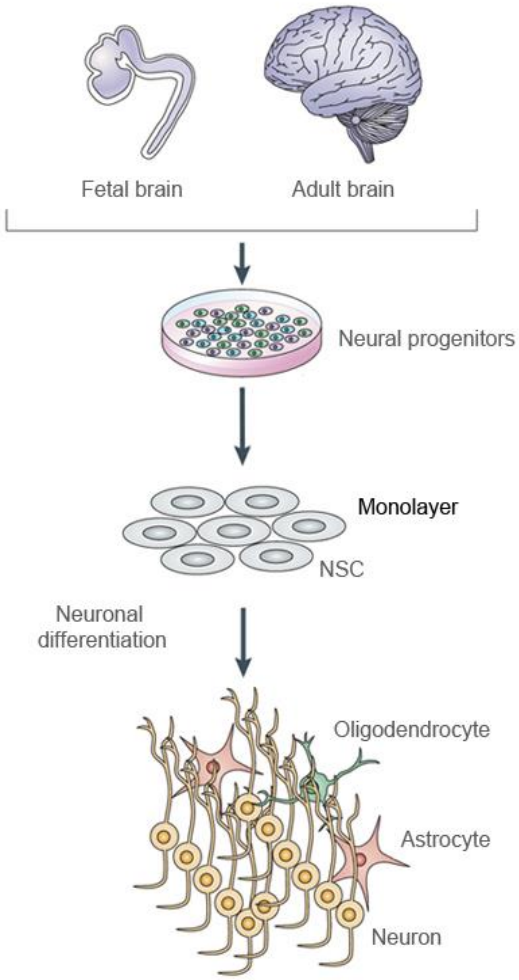


Figure 1.4. Monolayer culture system of neural stem cells. *Monolayer NSC lines can be derived from fetal and adult brains and display multipotency, generating neurons, astrocytes and oligodendrocytes under appropriate culturing conditions. The cellular composition of the monolayer NSC culture system is highly homogeneous. This property of monolayer NSC lines results in higher reproducibility of differentiation protocols and higher neurogenic potential. Adapted from Conti and Cattaneo, 2010.*

Neurogenesis, the process of generating integrated neurons from progenitor cells, was traditionally believed to occur only during embryonic stages in the mammalian CNS. Only recently neurogenesis was shown to also occur in the adult mammalian brain (Alvarez-Buylla and Garcia-Verdugo, 2002; Christie and Cameron, 2006; Gage, 2000; Pignatelli et al., 2009).

Neurogenesis in mammals begins with thickening of the ectoderm, leading to the induction of neuroectoderm, which forms the neural plate, at E7.5 in mice, and

then folds to give rise to the neural tube. These structures are made up by a layer of neuroepithelial cells (Götz and Huttner, 2005), which contact both apical (ventricular) and basal (pial) surfaces. Initially, neuroepithelial cells divide symmetrically at the ventricular surface to increase the pool of stem cells. However, at a certain point, they start dividing asymmetrically, generating stem cells that remain in the ventricular zone, and other cells that migrate radially outward (Haubensak et al., 2004). These cells are responsible for the first wave of neurogenesis in the neural tube, after which they give rise to radial glia (RG) (Conti and Cattaneo, 2010).

The name of RG was attributed due to several characteristics shared with astrocytes, including the presence of glycogen granules and the expression of glial fibrillary acidic protein (GFAP) (Choi and Lapham, 1978; Levitt and Rakic, 1980), astrocyte-specific glutamate transporter (Shibata et al., 1997) and brain lipid binding protein (Feng et al., 1994). RG cells act as guide wires for the migration of neurons (Malatesta et al., 2008). It is now clear that in many regions of the CNS, RG also represents the main population of neural progenitors and that their progeny includes neurons, astrocytes, oligodendrocytes, ependymocytes and adult NSCs (Conti and Cattaneo, 2010; Malatesta et al., 2003; Merkle et al., 2004; Spassky et al., 2005). While in many species RG persists into adulthood, in the mammalian brain RG disappears soon after birth, giving rise to the adult astrocyte-like NSCs (Merkle and Alvarez-Buylla, 2006).

1.2.1. Adult NSCs

The existence of adult NSCs, capable of self-renewing and generating both neuronal and glial derivatives has been an area of great controversy. Adult NSCs were first isolated from the adult CNS of rodents (Reynolds and Weiss, 1992), and later from humans (Kukekov et al., 1999). Full acceptance of postembryonic mammalian neurogenesis was not immediate until Luskins *et al.* (Luskin, 1993) described neurogenesis in the anterior portion of the subventricular zone (SVZ) in postnatal mice, Lois and Alvarez-Buylla (Lois and Alvarez-Buylla, 1993) demonstrated neurogenesis in the adult mouse SVZ, and several groups showed neurogenesis in the subgranular layer of the hippocampus (Altman and Das, 1965; Cameron et al., 1993; Kaplan and Bell, 1984; Kuhn et al., 1996). The discovery of the "no new neuron" dogma suggested that ongoing adult neurogenesis might be supported by a population of multipotent NSCs. This idea was based on the

following observations: (1) primary precursors within CNS germinal zones continue to produce neurons and glia throughout an organism life; and (2) cells from the adult brain with properties of NSCs can be grown *in vitro* with growth factors.

Along the lateral walls of the lateral ventricles lies the largest germinal zone of the adult mammalian brain, the SVZ. NSCs in the SVZ give rise predominantly to committed progenitor cells that migrate interiorly into the olfactory bulb (OB) via the rostral migratory stream (Kornack and Rakic), where they differentiate into two kinds of local olfactory interneurons, granule and periglomerular cells (Altman, 1969; Kornack and Rakic, 2001; Luskin, 1993). SVZ NSCs can be dissociated and grown in culture as free-floating spheres, or neurospheres, in the presence of epidermal growth factor (EGF), fibroblast growth factor (FGF), or combination of both growth factors (Gage, 2000; Temple and Alvarez-Buylla, 1999; Weiss et al., 1996). Some of these primary spheres are capable of generating other spheres upon dissociation and can be renewed through many passages.

The hippocampus is other major site of neurogenesis in adult mammals. Granule neurons in the dentate gyrus (DG) are born locally in the sugranular zone (SGZ). The progenitors in the SGZ migrate into the granular cell layer (Kuhn et al.) and also differentiate into neurons (Eriksson et al., 1998).

In the SVZ, the NSC pool is larger than in the SGZ (Yao et al., 2012), and three types of progenitors can be found, defined by their morphology, ultrastructure, and molecular markers: SVZ neuroblasts (type A cells) migrate in homotypic chains (Lois et al., 1996) into to OB through the RMS, where they differentiate into several types of neurons, depending on their place of origin within the SVZ (Lois and Alvarez-Buylla, 1994; Merkle et al., 2007). These chains are unsheathed by slowly proliferative type B cells (Lois et al., 1996). B cells have properties of astrocytes, including light cytoplasm, thick bundles of GFAP-positive intermediate filaments, gap junctions, glycogen granules, and dense bodies (Doetsch et al., 1999), which have been hypothesized to represent the primary neuronal precursors *in vivo* (Mu et al., 2010). Scattered along the chains of type A cells are clusters of spherical and rapidly dividing C cells. The SVZ is largely separated from the ventricle cavity by a layer of ependymal cells. In contrast to the SVZ, the sugranular layer is deep within the brain at the interface of the GCL of the DG and the hilus of the hippocampus and, opposite

to the extensive tangential migration undertaken by OB neurons, hippocampal granule neurons move only a short distance into the GCL (Seri et al., 2001). The NSCs from this region are radial astrocytes, also known as RG-like cells or type 1 progenitors. Although expressing the astrocyte marker GFAP, these cells are morphologically and functionally different from mature astrocytes.

Recently, type 1 cells were also shown to be indeed multipotent NSCs, as they are capable of undergoing self-renewing symmetric and asymmetric divisions and can differentiate into both neurons and astrocytes (Bonaguidi et al., 2011). Radial astrocytes have long radial processes that penetrate the GCL. Dividing radial astrocytes generate immature, non-radial intermediate progenitors termed type 2 or type D cells. These D cells can differentiate into neuroblasts, which migrate into the nearby granule layer and differentiate into glutamatergic neurons (Kuhn et al., 1996). D cells developed a branched apical process that becomes the dendrites of the new granule neuron, and later an axon that projects to area CA3 (Stanfield and Trice, 1988). Approximately half of these cells survive and integrate the pre-existing neuronal circuits (Toni et al., 2007; van Praag et al., 2002). The demonstration of neurogenesis in adult human brains not only proves the unforeseen regenerative capacity of CNS, but also gives hope for strategies to repair damaged adult CNS after injury of neurodegenerative diseases.

1.2.3. Apoptosis-associated factors in neural differentiation

The mechanisms controlling the neural differentiation process remain poorly understood, and many molecules and their specific functions are yet unknown. Emerging evidence suggests that apoptosis players and inducers may also control cell fate, without involving cell death *per se* (Fernando et al., 2002; Fernando and Megeney, 2007; Galluzzi et al., 2012). In fact, essential players of apoptotic executioner pathways cannot be totally blocked to assure differentiation efficiency but, at the same time, must be carefully regulated to avoid cell loss (Chasis and Schrier, 1989; Solá et al., 2012). Specifically, apoptosis-related microRNAs (miRNAs), such as miRNA-34a, -16 and let-7a, and proteins, including caspases, calpains, and Bcl-2 and p53 family members are involved in mouse NSC differentiation by mechanisms independent of cell death (Aranha et al., 2011; Aranha et al., 2010; Aranha et al., 2009; Solá et al., 2011a).

1.2.3.1. Proteases

Caspases are cysteinyl aspartate-specific proteases with a number of properties that make them highly effective as cellular remodeling enzymes. Although their critical function in apoptosis is firmly established, caspase activation is also essential for dynamic, non-apoptotic cell processes, such as innate immune response, neural regeneration, NSC fate determination, and neural activation. Indeed, caspases have recently been implicated in the differentiation process for a variety of model systems, including neural cells (Santos et al., 2012b; Schwerk and Schulze-Osthoff, 2003). It has been demonstrated that NSC differentiation depends on endogenous caspase-3 activity that may influence kinase activities associated with changes in phenotype (Fernando et al., 2005). In addition, caspases modulate FOXO3A/inhibitor of differentiation 1 (Id1) signaling during mouse NSC differentiation (Aranha et al., 2009). In fact, caspase inhibition and silencing of p53 synergistically delayed neural differentiation, with no evidence of apoptosis. Recently, the role of caspases in neural differentiation has been further elucidated and suggested to be required for neuronal growth cones (Campbell and Holt, 2003), learning and memory (Lu et al., 2002), axon routing, synaptic connections (Ohsawa et al., 2010), among others. Several mechanisms for regulating non-apoptotic caspase function in neural differentiation have been proposed, including transient caspase activation, local caspase activation and regulation of endogenous caspase inhibitors (Solá et al., 2012).

Although the available literature on calpain activity in NSCs is scarce, recent data has also elucidated the role of calpain 1 and 2 during NSC self-renewal and differentiation. Santos *et al.* (Santos et al., 2012b) showed that calpain 1 maintains stemness and represses neural differentiation, while calpain 2 acts as potential modulator of gliogenesis. These results underscored the distinct regulatory functions of calpain 1 and 2 in NSC fate decision.

1.2.3.2. Bcl-2 family

Bcl-2 family proteins suffer developmental regulation in the nervous system, being highly expressed in neuroepithelial cells of the SVZ, and in post-mitotic neurons before adulthood (Merry et al., 1994). Bcl-2 acquired immunoreactivity signal in the brain of squirrel monkeys, specifically in areas involved in neurogenesis and morphogenesis (Bernier and Parent, 1998). Therefore, it has been suggested that Bcl-2 might be important, but not crucial, during neuronal cell differentiation.

In vitro, Bcl-2 expression was associated with both neural differentiation of human neural-crest-derived tumor cell line, Paju (Zhang et al., 1996), and RA (Sarkar and Sharma)-mediated differentiation of CNS neurons (Wang et al., 2001). In PC12 cells, in turn, Bcl-2 was shown to trigger differentiation as a result of prevention of cell death induced by serum free medium (Sato et al., 1994). In addition, other studies indicate that Bcl-2 may act as an intercellular factor that controls neurite extension and axonal regeneration (Eom et al., 2004; Suzuki and Tsutomi, 1998). It was recently reported that Bcl-x_L and Bax play an instructive role in determining the fate of embryonic cortical precursor cells (Chang et al., 2007). Bcl-x_L and Bax appear to mediate neurogenesis and astrogliogenesis, respectively, independently from their roles in cell survival and apoptosis (Solá et al., 2012). These findings support the possibility that determination of neuronal versus astroglial lineages is actively regulated by balanced levels of anti- and pro-apoptotic Bcl-2 family proteins in NPCs (Chang et al., 2007). Finally, pro-apoptotic Bim was associated with the neurogenesis of adult-born NPCs (Bunk et al., 2010).

1.2.3.3. p53 family

p53 family members, p53, p63 and p73 play crucial roles as regulators of NSC proliferation and differentiation (Murray-Zmijewski et al., 2006).

p53

In the past several years, a specific novel function for p53 in neuronal biology has emerged. In fact, increased evidence points to a role for p53 in neural progenitor proliferation and differentiation, independent of its apoptotic role (Solá et al., 2013; Tedeschi and Di Giovanni, 2009). p53 was shown to be required to the differentiation of mouse embryonic stem cells (ESCs) by suppressing expression of Nanog, a transcription factor essential to the maintenance of pluripotency of ESCs (Chen et al., 2008; Mitsui et al., 2003) (Fig. 1.5). In addition, it was also reported that in neurons from a $p53^{-/-}$ mouse, p53 plays an important role in neuronal differentiation (Ferreira and Kosik, 1996). Curiously, high levels of p53 mRNA have also been detected in the developing brain in areas showing little or no apoptosis. Some p53-null embryos die during development, and a percentage of the surviving mice exhibit neuronal abnormalities, namely defects in neural tube closure (Armstrong et al., 1995), resulting in exencephaly

(Sah et al., 1995). Curiously, this exencephalic phenotype is identical to that observed in animals with mutations in other members of the intrinsic death pathway, including Apaf-1, caspase 3 (Kuida et al., 1996), and caspase 9 (Kuida et al., 1998). p53 was already shown to promote neurogenesis through downregulation of the Akt/p-FOXO3A/Id1 pathway (Aranha et al., 2009) (Fig. 1.5). Furthermore, the role of p53 during mouse NSC differentiation has been further dissected by other studies (Solá et al., 2012).

p53 might also play a key role in neural differentiation as a limiting factor of stem cell proliferative competence (Armesilla-Diaz et al., 2009; Krizhanovsky and Lowe, 2009). Recent experiments of NSCs extracted from the OB of wild-type and p53-null mouse embryos showed that p53 controls proliferation, chromosomal stability, and differentiation pattern of mouse OB stem cells (Armesilla-Diaz et al., 2009). In fact, p53-null NSCs have increased proliferation with tendency to a neuronal phenotype, with concomitant reduction of astrocytes. This effect had been previously reported in SVZ-derived stem cells from adult mice (Gil-Perotin et al., 2006). Studies in neuronal-like PC12 and neuroblastoma cells have also suggested that p53 plays a role in cell survival following NGF administration (Hughes et al., 2000; Zhang et al., 2006a), and that the interaction of p53 with neuronal-specific transcription factors, such as Brn-3a, may determine a shift from cell death to neuronal survival (Hudson et al., 2005; Hudson et al., 2004). In fact, when forming a complex with Brn-3a, p53 cannot activate pro-apoptotic genes, such as Bax and Noxa, but rather shows increased affinity for the pro-differentiation gene p21. p53 was also reported to bind to the NGF receptor TrkA, which is required for PC12 neuronal differentiation (Browes et al., 2001), and to activate TrkA expression and consequent MAPK pathways (Zhang et al., 2006a) (Fig. 1.5). Also in PC12 cells, p53 regulates the expression of both actin-binding protein Coronin 1b and the GTPase Rab13, which are needed for correct neurite outgrowth and maturation of cortical neurons *in vitro* (Di Giovanni et al., 2006; Tedeschi and Di Giovanni, 2009) (Fig. 1.5). Integrated transcriptomic profiling, *in silico* promoter analysis, and functional studies of murine NSCs established that inactivation of both p53 and phosphatase and tensin homolog (PTEN) promotes an undifferentiated state, with high self-renewal potential, and increased Myc protein levels (Zheng et al., 2008). In agreement, increased Myc activity was validated as a potent contributor to impaired

differentiation and enhanced self-renewal of NSCs in murine double-null mice for p53 and PTEN. Thus, due its dual actions on cell cycle and cell death, p53 plays a versatile role in the regulation of cellular growth and differentiation, being considered a gatekeeper of self-renewal in NSCs that may function either as a pro-apoptotic or differentiation factor in neural precursors.

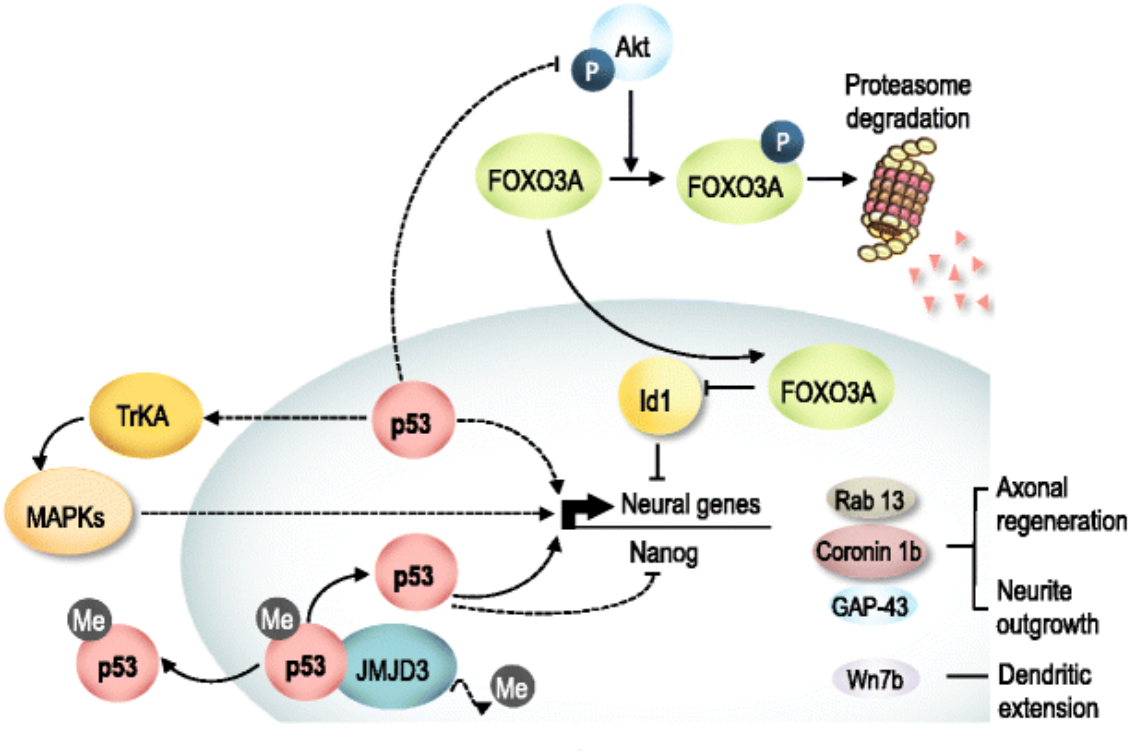


Figure 1.5. p53 in neurogenesis. *p53 is involved in non-apoptotic processes such as cell-fate determination and actin-cytoskeleton reorganization. p53 plays a critical role in neuronal differentiation, in part via regulation of TrkA and MAPK pathways in PC12 cells. Moreover, Wnt7b is a new putative p53 target during NGF-mediated PC12 neuronal differentiation, involved in dendritic extension. p53 also regulates the expression of actin-binding protein Coronin 1b and the GTPase Rab13 in primary cultured neurons, both of which associate with the cytoskeleton and regulate neurite outgrowth, as well as the expression of the axonal growth-associated protein GAP-43, which is involved in pro-axon outgrowth and regeneration protein. In mouse NSC, p53 promotes neurogenesis by downregulating the Akt/p-FOXO3A/Id1 proliferation pathway. Interestingly, p53 and the desmethylase JMJD3, a key regulator of neurogenesis, are coordinately regulated during adult neural differentiation. JMJD3 directly interacts with p53 to induce demethylation of p53 and subsequent p53 nuclear accumulation during specific stages of neural differentiation. Adapted from Solá et al. 2012.*

Epigenetic mechanisms have an important role in control maintenance of stem cell state and in the differentiation process (Li and Zhao, 2008; Wu and Sun, 2006). In this regard, during the past decades, covalent modifications have been

discovered to play fundamental roles in either homeostatic maintenance or stress-induced activation of p53.

Specific targets and cofactors of p53 during neuronal differentiation may be unique and different from those regulated in genotoxic stress and apoptosis. This is possible by the multiple PTMs that target p53 on its N- and C-termini, which are not limited to phosphorylation, but may also include acetylation, sumoylation, neddylation, ubiquitination, or methylation (Collavin et al., 2010; Lavin and Gueven, 2006; Solá et al., 2011). In fact, specific combination of PTMs differentially regulates the interaction of p53 with co-activators and co-repressors and produces distinct gene expression profiles, which may also dictate cell fate.

p53 PTMs directly affect the transcriptional activity of p53 and regulate its affinity to several cofactors, which in turn regulate the occupancy of p53 specific promoters (Sims and Reinberg, 2008). Phosphorylation at Ser 15, Ser 20, Ser 33, Ser 46 and Thr 18 promotes p53 stabilization by preventing either its nuclear export or its recruitment to specific promoters. Early in the differentiation of ESCs, phosphorylation of p53 at Ser 15, Ser 315 and Ser 392 is significantly increased, which is thought to activate p53 (Xu, 2003). p53 phosphorylation at Ser 315 was also shown to be important for p53-dependent suppression of Nanog during differentiation of ESCs (Lin et al., 2005). The acetylation of p53 in neurons is mediated by the activity of at least these two histone acetyltransferases, CREB-binding protein (CBP)/p300 and p300/CBP-associated protein (P/CAF). CBP/p300 acetylates Lys370, Lys372, Lys373 and Lys382, and P/CAF acetylates only Lys320 (Brooks and Gu, 2003). These kinase and acetyltransferase pathways are activated downstream from NGF and BDNF signaling during neuronal differentiation and axon outgrowth. Interestingly, the acetylation of p53 at Lys 320 leads to increased transcriptional activation of the p21 promoter, which triggers G1/S arrest and promotes neuronal differentiation in PC12 cells (Wong et al., 2004). Interestingly, p53 acetylation in the same residue was reported to be involved in promotion of neurite outgrowth (Di Giovanni et al., 2006). Additionally, acetylated p53 at Lys372, Lys373 and Lys382 was shown to drive axon outgrowth and GAP43 expression, and to bind to specific elements on the neuronal GAP43 promoter (Tedeschi and Di Giovanni, 2009) (Fig. 1.5).

p53-induced control of cell fate may also be associated with p53 subcellular distribution. In fact, it has been reported that p53 translocates to the nucleus

following induction of differentiation (Brynczka and Merrick, 2007; Solá et al., 2011). SIRT1, a p53 deacetylase, inhibits p53 nuclear translocation in mouse ESCs and inhibits p53-mediated suppression of Nanog expression (Han et al., 2008). Moreover, p53 demethylation appears to be also essential in p53 nuclear distribution in mouse NSC differentiation, associated with an increase in neuronal markers (Solá et al., 2011b). In fact, recent studies have already established protein methylation as a novel mechanism of p53 regulation (Scoumanne and Chen, 2008). Recently, it has been shown that this proapoptotic protein cross-talks with key regulators of neurogenesis, such as the histone H3 lysine 27-specific demethylase JMJD3. In fact, Solá *et al*. have demonstrated that JMJD3 increases total levels of p53 by a mechanism dependent, at least in part, of ARF, a mechanism that is not associated with cell death. Importantly, they have also shown that JMJD3 directly interacts with p53 to modulate p53 methylation levels and regulate p53 cellular distribution during mouse neural differentiation (Solá et al., 2011) (Fig. 1.5). The molecular crosstalk between the transcription factor p53 and the histone lysine specific demethylase 1 has been previously demonstrated by Huang *et al.* (Huang et al., 2007).

While p53 is ubiquitously expressed, p63 exhibits a tissue-specific distribution that it is most detectable in embryonic ectoderm and in basal regenerative layers of stratified epithelial tissues in the adult (Yang et al., 1998). However, both TA and ΔNp63, as well as TA and ΔNp73 proteins are also expressed in the cortex of embryonic, postnatal, and adult brains (Jacobs et al., 2005).

p73

p73 is known to play an important role in controlling neuronal development, being an essential regulator of NSC maintenance and differentiation in both embryonic and adult neurogenesis (Fujitani et al., 2010; Talos et al., 2010). The first evidence for the role of p73 in neural differentiation of a normal mammalian cell came from studies showing that both p53 and p73, but not p63, are involved in thyroid hormone-induced oligodendrocyte precursor cells differentiation, and that p73 also plays a crucial role in platelet-derived growth factor withdrawal-induced differentiation (Billon et al., 2004).

p73 developmental role is supported by the phenotype of total p73 KO mice. Unlike p53 null mice, total p73 KO mice do not develop tumors; however, they show developmental defects in the CNS with 100% penetrance (Yang et al., 2000),

including congenital hydrocephalus and hippocampal dysgenesis, with loss or truncation of the lower blade of the neurogenic DG (Talos et al., 2010), thinning of the SVZ in the forebrain and loss of the Cajal-Retzius neurons (Hernández-Acosta et al., 2011). The total p73 KO mice also have defects in both embryonic and adult neurogenesis, suggesting that p73 isoforms may be important factors for the maintenance of NSCs (Fujitani et al., 2010; Holembowski et al., 2011; Talos et al., 2010). $p73^{-/-}$ mice also have a reduction in cortical thickness as a consequence of loss of mature cortical neurons (Pozniak et al., 2002). Curiously, ΔNp73 variants are the predominant expression product of the p73 gene in developing and adult tissues. ΔNp73 isoforms are expressed in postmitotic neurons and act as survival factors of differentiated mature postmitotic neurons in discrete regions of the brain (Tissir et al., 2009; Wilhelm et al., 2010). Their loss explains this neurological phenotype (Pozniak et al., 2000). In addition selective TAp73 null mice also show hippocampal dysgenesis with loss of the lower blade of the DG, a phenotype intermediate between the phenotypes of $p73^{-/-}$ and $p53^{-/-}$ mice (Tomasini et al., 2008), reflecting a reduction in neurogenesis in the SGZ of the DG, and suggesting that TAp73 may be required for NSC proliferation (Michalak et al., 2008). Therefore, these studies suggest a role for p73 in neurogenesis and a cooperation or redundancy between the isoforms during this process. p73 also maintains an adequate neurogenic pool by promoting self-renewal and proliferation and inhibiting premature senescence of NSC and early progenitor cells in both embryonic and adult neurogenesis (Talos et al., 2010). Curiously, TAp73 appears to act via the basic helix-loop-helix Hey2 to promote long-term maintenance of neural precursors (Fujitani et al., 2010). Further, p73 deficiency results in impaired self-renewal and premature neuronal differentiation of mouse NPCs independently of p53 [103].

Neuroblastoma cell differentiation *in vitro* requires activation of a molecular pathway by p73, which is not shared by p53. p73 protein increases in cells induced to differentiate by RA (De Laurenzi et al., 2000),. Exogenously expressed p73, in turn, is sufficient to induce neurite outgrowth, expression of neurofilaments and neural adhesion molecule, (N-CAM), downregulation of N-Myc and upregulation of pRb, all markers of neural differentiation (De Laurenzi et al., 2000). An *in vitro* neurosphere assay from *p*73 KO mice and their wild-type counterparts, demonstrated that p73 KO results in decreased capacity to form neurospheres, and smaller neurosphere sizes with fewer cells per neurosphere, indicating an impairment in the proliferation of

progenitors (Talos et al., 2010). Together, these results indicate that p73-deficient NSCs are not only deficient in self-renewal but also in proliferation and generation of progeny. Finally, p73 drives the expression of miRNA-34a in mouse cortical neurons, which then modulates the expression of synaptic targets, including synaptotagmin-1 and syntaxin-1A (Agostini et al., 2011b). In fact, p73 was shown increased during postnatal development of the brain, when synaptogenesis occurs (Agostini et al., 2011a).

p63

The p63 protein, in turn, has been extensively described as a key regulator of development and maintenance of stratified epithelial tissues, having a tissue-specific distribution, mostly detectable in the basal layer of epithelia, including epidermis (Yang et al., 1998). In fact, the phenotype of *p63*-deficient mice presents several developmental defects, suggesting that the primary biological function of p63 proteins is to regulate development. However, brain malformations have not been reported in humans with p63 mutations. This phenotype contrasts with mice lacking p73 gene, which have immunological, neurological, and pheromone defects (Yang et al., 2000). This raises the question whether p63 has a similar important role in neural differentiation as p53 and p73. Thus far, it is not clear whether p63 contributes to brain development. Dugani *et al.* (Dugani et al., 2009) have reported that p63 is a crucial pro-survival regulator of NPCs. Jacobs *et al.* (Jacobs et al., 2005) have also investigated the role of p63 in developing sympathetic neurons of the autonomous nervous system, concluding that the TAp63 isoforms play pro-apoptotic functions in these neurons. Furthermore, during embryonic stages, p73 and p63 are co-expressed in Cajal Retzius cells derived from a marginal zone of developing cortex, the cortical hem, which is a putative signaling center and the main place for the generation of CR cells. However, in contrast with the previous studies, the analysis of $p63^{-/-}$ and $p73^{-/-}$ mice shows that only p73 is essential for brain development and CR cell formation (Tissir et al., 2009), indicating that p63 expression and signaling in the cortical hem seems to be an accessory feature of these cells, and not essential for brain development in general (Hernández-Acosta et al., 2011).

Despite these divergences, it has been reported that p63 expression during development of the CNS is scant. Indeed, TAp63 and ΔNp63 mRNA levels are very low in murine embryonic cortex (Holembowski et al.; Jacobs et al., 2005), which is in

contrast to the essential role of p73 in neurogenesis (Agostini et al., 2010; Fujitani et al., 2010; Holembowski et al., 2001; Talos et al., 2010). In the SVZ of the lateral ventricle of postnatal mice, the three members of the p53 family are detected and may influence cell fate in this region of the adult brain. However, in contrast to p73, the detection levels of p63 and p53 were weak. In fact, immunohistochemistry revealed co-expression of p73 and a marker of proliferating cells, the proliferating cell nuclear antigen (Uberti et al.) in the SVZ, where the same double staining was not possible with either p63 or p53 (Hernández-Acosta et al., 2011; Lois and Alvarez-Buylla, 1993). Nevertheless, in contrast to p63-restricted expression observed during brain development, high levels of p63 were visualized in adult human cerebral cortex and hippocampus (Hernández-Acosta et al., 2011), suggesting a role for p63 in the adult brain. Furthermore, in postnatal mice, p63 is expressed in neurogenic niches, such as the SVZ of the lateral ventricle (Hernández-Acosta et al., 2011). This suggests that p63 may have a role during adult neurogenesis that remains to be explored.

1.2.4. Role of Aβ peptides in neural differentiation

Aβ peptides are highly multifunctional identities, which may display significant non-pathological activities (Fig. 1.6). Indeed, proposed functions of Aβ peptides include trophic and neuronal survival, neural differentiation, control of synaptic activity and memory consolidation, cholesterol transport and antioxidant functions (Parihar and Brewer, 2010; Pearson and Peers, 2006; Plant et al., 2003). The Aβ cellular functions are evident only when the peptides are present at physiological concentrations, before they aggregate and form amyloid fibrils (Parihar and Brewer, 2010; Pearson and Peers, 2006; Plant et al., 2003; Ramsden et al., 2002). In fact, Plant *et al.* (Plant et al., 2003) showed that, in rodent and human neuronal cells, *in vitro* inhibition of γ- or β-secretase culminates with toxicity. Recovery of cell viability using physiologically concentrations of exogenous Aβ suggests that this peptide may have a role in the normal function of neuronal cells as suggested previously by Ramsden and co-workers (Ramsden et al., 2001). Recent studies indicate that Aβ plays an important role at the synapse as well as in synaptic structure and functional plasticity, critical to learning and memory (Parihar and Brewer, 2010). A necessary role of Aβ in synaptic plasticity and memory in normal brain is also supported by the observation that APP KO mice show impaired LTP and memory (Dawson et al., 1999). The impaired synaptic plasticity and memory found in beta-

site APP-cleaving enzyme 1 KO mice also suggest a crucial role of Aβ (Laird et al., 2005).

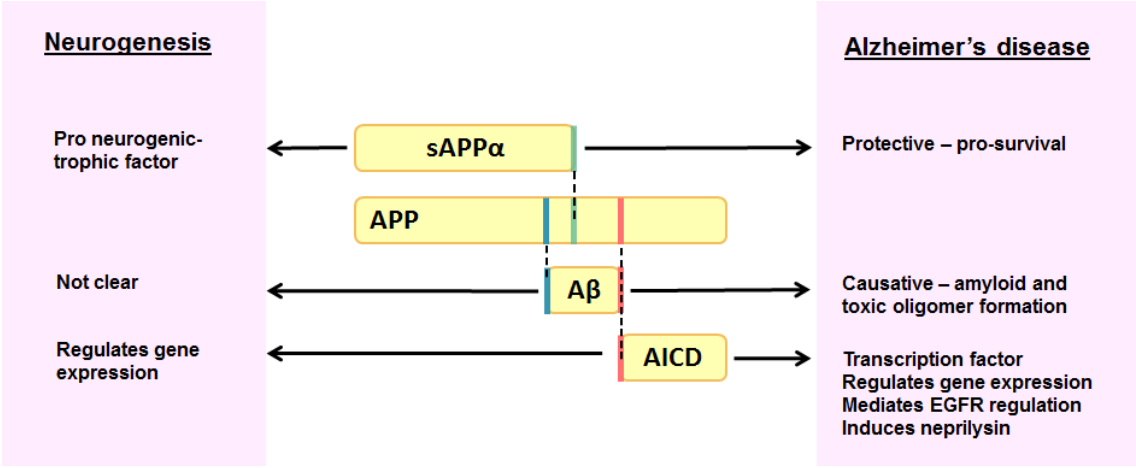


Figure 1.6. Pathological versus proneurogenic effects of amyloid β peptides. *A summary of suggested function of β- and γ-secretases-generated APP fragments, including Aβ peptides, in AD neuropathology and in neurogenesis. sAPPα, soluble amyloid precursor protein α. Adapted from Lazarov and Marr, 2010.*

The evidence that Aβ is present in the CSF of healthy individuals and in media from neuronal cell cultures (Blennow and Zetterberg, 2009; Tamaoka et al., 1997) indicates that Aβ may also play a role in the normal physiology of the CNS. Importantly, it was already reported that non-aggregated peptide at low doses exhibits neurotrophic effects including enhancement of neuronal survival and neurite-promoting effects (Luo et al., 1996; Pike et al., 1991; Whitson et al., 1990; Yankner et al., 1990). Indeed, $A\beta_{1-42}$ monomers were shown to promote the survival of developing neurons under conditions of trophic deprivation, and protect mature neurons against excitotoxic death (Giuffrida et al., 2009). In addition, $A\beta_{1-42}$ increased the number of newborn neurons in cultured NSCs (Chen and Dong, 2009; López-Toledano and Shelanski, 2004). Neurotrophic function of $A\beta_{1-40}$ was obtained in a cell culture treated with picomolar and micromolar levels of $A\beta_{1-40}$ (Chen and Dong, 2009; Plant et al., 2003; Yankner et al., 1990). These findings provide compelling evidence for a role for Aβ in neuronal survival.

Recently, *in vitro* and *in vivo* studies revealed that Aβ is also able to drive the differentiation of NPCs toward neurons (Calafiore et al., 2006; Chen and Dong, 2009; Heo et al., 2007; López-Toledano and Shelanski, 2004; Sotthibundhu et al., 2009). However, the precise role of different forms of monomer, oligomer, and fibrillar Aβ

on the neurogenesis are still poorly understood. Indeed, there is a lot of variance in the literature regarding the experimental conditions used to address the neurogenic effects of Aβ. The experiments differ, not only in the form of Aβ used, but also in the type of NSC culture used, and in the concentrations of Aβ incubated (Calafiore et al., 2012; Haughey et al., 2002a; Haughey et al., 2002b; Heo et al., 2007; Kim et al., 2007; López-Toledano and Shelanski, 2004; Sotthibundhu et al., 2009). In cultures of NSCs derived from striatum and hippocampus it was demonstrated that Aβ has a role on the survival and neuronal differentiation (López-Toledano and Shelanski, 2004). The authors showed that this peptide increases the total number of neurons *in vitro* in a dose-dependent manner. In addition, they have shown that neurogenesis is induced by $A\beta_{1\text{-}42}$ but not by $A\beta_{1\text{-}40}$ or $A\beta_{25\text{–}35}$, and that the pro-neurogenic activity appears to be a property of Aβ oligomers and not fibrils. In concordance, other studies showed that low micromolar concentrations of oligomeric $A\beta_{1\text{-}42}$ enhance neuronal differentiation of adult NSCs and their ability to migrate (Heo et al., 2007). An *in vivo* study also demonstrated that $A\beta_{1\text{-}42}$ promotes amyloid deposition while $A\beta_{1\text{-}40}$ inhibits it. Chen *et al*. (Chen and Dong, 2009) have reported notable neurogenic and survival effects of soluble $A\beta_{1\text{-}40}$ in primary NPCs; $A\beta_{1\text{-}42}$ treatment both *in vitro* and *in vivo*, as well as endogenous generation of Aβ in transgenic models of AD, stimulated neurogenesis of young adult SVZ precursors (Sotthibundhu et al., 2009). The neurogenic effect of $A\beta_{1\text{-}42}$ was found to require expression of the $p75^{NTR}$ by the precursor cells, and activation of $p75^{NTR}$ by metalloprotease cleavage. In neurospheres incubated with oligomers of synthetic $A\beta_{1\text{-}42}$, the expression levels of DNA polymerase-β, an enzyme that represses proliferation and promotes differentiation, were rapidly increased (Calafiore et al., 2012).

On the other hand, studies using animal models of AD have proposed the opposite effect of Aβ peptides in neurogenesis (Haughey et al., 2002a; Haughey et al., 2002b). In fact, it has been reported that Aβ compromises the survival and differentiation processes (Haughey et al., 2002b; Mazur-Kolecka et al., 2006; Millet et al., 2005). However, these results appear to be correlated with the decreased levels of soluble Aβ peptide observed in AD. Consistent with this, others have demonstrated that higher levels of Aβ monomers, oligomers, and fibrils in the brain of individuals with amyloidosis-β induce oxidative stress and do not promote adult endogenous neurogenesis (Mazur-Kolecka et al., 2006). AD is a dynamic disease

process, and it is plausible that an increase in neuronal differentiation is followed by an overall decline as Aβ aggregates and loses its neurogenic functions.

1.2.5. Autophagy during neural differentiation

Autophagy acts as a quality control mechanism in differentiated cells by promoting basal turnover of long-lived proteins and organelles, and selectively degrading damaged cellular components (Wirawan et al., 2012). In addition, autophagy promotes morphological and structural remodeling (Singh et al., 2009). In fact, as a dynamic and highly inducible catabolic process, it can drive the rapid cellular changes necessary for proper differentiation and/or development.

In mammals, autophagy plays key roles in pre-implantation embryonic development (Levine and Ranganathan, 2010; Tsukamoto et al., 2008), survival during neonatal starvation (Aburto et al., 2012; Mizushima and Levine, 2010), cancer (Chen and Karantza, 2011; Edinger and Thompson, 2003), neuronal degeneration (Hara et al., 2006; Komatsu et al., 2006; Wong and Cuervo, 2010), and cell differentiation during erythropoiesis, lymphopoeisis, adipogenesis (Asanuma et al., 2003; Mizushima and Levine, 2010; Pua et al., 2007; Vessoni et al., 2012), and neurogenesis (Aburto et al., 2012; Zeng and Zhou, 2008; Zhao et al., 2010) (Fig. 1.7) Targeted mutagenesis of a few Atg genes in mice substantiated these hypotheses. Severe neural tube defects and embryonic lethality were associated with impaired autophagy in mice lacking the autophagy regulator Ambra1 (Cecconi et al., 2007; Fimia et al., 2007). In embryos homozygous for the gene-trapped (gt) allele Ambra1$^{gt/gt}$, it was observed reduced expression of neurogenic genes, accumulation of ubiquitinated proteins, unbalanced cell proliferation and excessive apoptotic cell death (Fimia et al., 2007). These interesting results suggest that autophagy may also be involved in the proliferative state or in the maintenance of pluripotency of NPCs. Activation of autophagy was also shown to be required for differentiation of glioma progenitor cells (Zhang et al., 2006b). It was shown that the autophagy inhibitors bafilomycin and 3-methyladenine could inhibit differentiation, while the autophagy activator and mTOR inhibitor, rapamycin, could promote it (Zhao et al., 2010). The basal low autophagic activity in glioma progenitor cells appears to be correlated with the inhibition of differentiation.

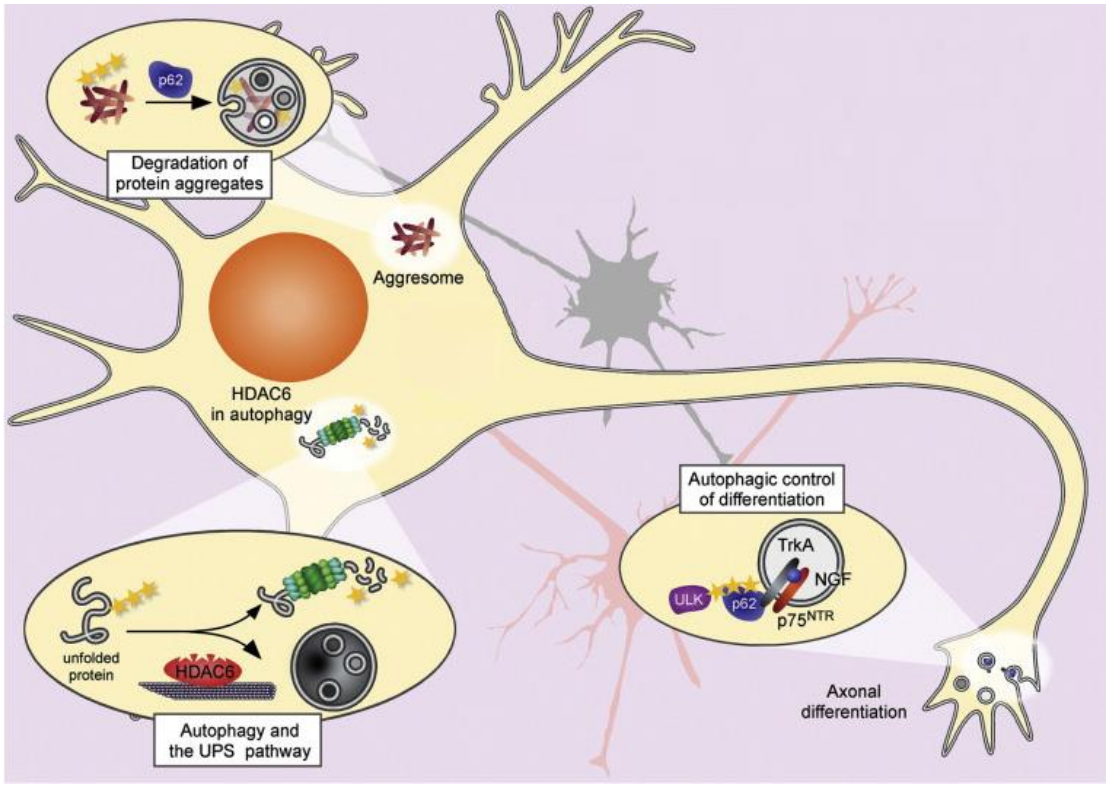


Figure 1.7. Autophagy is involved in neural differentiation. *Autophagy is involved in multiple aspects of neuronal physiology, including axonal differentiation and maintenance, UPS-independent removal of unfolded cytosolic proteins and elimination of aggregosomes. HDAC6, histone deacetylase 6. Adapted from Tooze and Schiavo 2008.*

Zhuang *et al.* (Zhuang et al., 2011) have also reported the relevance of autophagy in glioma-initiating cell differentiation and radiosensitization *in vitro* by rapamycin. The authors propose that autophagy deficiency in these cells contributes to the differentiation impairment and glioma radioresistance. NPCs from the mouse embryo olfactory bulb are also a well-characterized tool for the study of neuronal differentiation. Vazquez *et al.* (Vázquez et al., 2012) have recently investigated whether basal autophagy occurs in these cells using this *in vivo* model during development. In fact, in wild-type mouse OB, they observed a progressive increase in the expression of the autophagy genes Atg7, Beclin1 (the orthologue of yeast Atg6), LC3 and Ambra1, during the differentiation of mitral neurons *in vivo*, associated with upregulation of Ngn1, NeuroD and βIII-tubulin neurogenic markers. In addition, an increase of autophagic activity in NPCs from mouse embryo OB undergoing differentiation was also observed *in vitro* (Vázquez et al., 2012).

The requirement of autophagy for neural differentiation was further confirmed in Ambra1 haploinsufficient mice, where it was shown the generation of smaller neurospheres and fewer differentiated neurons (Vázquez et al., 2012). Importantly, these changes were reversed, to a large extent, by the metabolic substrate methylpyruvate, suggesting that in the context of neural differentiation, autophagy is crucial to energy supply, maintaining the ATP levels necessary for correct transition from proliferative precursor to postmitotic neuron. This hypothesis is indeed supported by previous studies in the developing retina, where autophagy was shown crucial for cell corpse clearance during naturally occurring cell death associated with neurogenesis (Mellén et al., 2008).

Objectives

The studies presented in this thesis were driven by the ambition to further understand the role of apoptosis in AD, and the function that AD-related apoptosis effectors may play in neural differentiation. Three main objectives were designed. First, our goal was to elucidate the precise role of p63 during Aβ-induced apoptosis and TUDCA protection. Second, we sought to investigate the potential involvement of p63 in regulating neural differentiation. Finally, we aimed to further explore the effect of soluble Aβ fragments in modulating neural proliferation and differentiation.

The specific questions addressed in this thesis are:

1. Does p63 play a role in Aβ-mediated cell death and/or TUDCA protection? Which molecular pathways does p63 modulate?
2. Does p63 modulate mouse NSC differentiation?
3. Can soluble Aβ peptides enhance neuronal-specific lineage of NSCs? How does Aβ regulate NSC fate determination?

The overarching goal of the research presented in this thesis is to increase our understanding on the pathogenic mechanisms of Aβ in AD as well as the role of apoptosis-regulatory players in NSC fate decision. The results may have broad implications in advancing our knowledge of neural degeneration and differentiation, and their interplay with the apoptosis machinery. Ultimately, our findings may prove useful in the comprehension of the key checkpoints responsible for neural degeneration *versus* differentiation, as an alternative to cell death, thus contributing to a better therapeutic management of apoptosis-associated AD and to a more efficient use of stem cells in neural-replacement therapy.

c-Jun Regulates the Stability of Anti-apoptotic ΔNp63 in Amyloid β-induced Apoptosis

Maria B. Fonseca[1], Ana F. Nunes[1], Cecília M. P. Rodrigues[1,2]

[a]*Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL), and*
[b]*Department of Biochemistry and Human Biology, Faculty of Pharmacy,*
University of Lisbon, Lisbon, Portugal

Journal of Alzheimer's Disease 2012; 28: 685–694

Reprinted from *Journal of Alzheimer's Disease* vol 28, Maria B. Fonseca, Ana F. Nunes, Cecília M. P. Rodrigues. c-Jun regulates the stability of anti-apoptotic ΔNp63 in amyloid-β-induced apoptosis, pages 685–694, Copyright 2012, with permission from IOS press. All rights reserved.

2.1. Abstract

p63, the structural and functional homologue of p53, is expressed either as a full-length isoform, containing a transactivation (TA) domain (TAp63), or as a truncated isoform, which lacks TA (ΔNp63). Amyloid β (Aβ) incubation of neuronal cells results in stress-induced cell death through poorly understood mechanisms. We investigated the role of p63 in Aβ-induced stress. Our results show that Aβ-induced apoptosis of rat PC12 neuronal-like cells and primary cortical neurons was associated with stabilization of pro-apoptotic TAp63 and, most importantly, degradation of anti-apoptotic ΔNp63 through a MAPK- and proteasome-dependent mechanism. This was associated with increased c-Jun, and partially modulated by tauroursodeoxycholic acid. As expected, classic genotoxic insults resulted in c-Jun upregulation and concomitant ΔNp63 reduction. Endogenous and ectopic ΔNp63 expression was also markedly reduced by c-Jun overexpression. Further, Aβ-mediated ΔNp63 degradation occurred in a c-Jun-dependent manner. Downregulation of c-Jun expression by specific c-Jun siRNA abrogated the reduction of ΔNp63 levels following Aβ insult, whereas overexpression of c-Jun led to its degradation. c-Jun significantly decreased ΔNp63 half-life. Together, these findings demonstrate that the abundance of anti-apoptotic ΔNp63 in response to Aβ-induced cell stress is regulated by a c-Jun-dependent mechanism, and highlight the importance of finding novel targets for potential therapeutic intervention.

Keywords: Amyloid β peptide; Apoptosis; c-Jun; ΔNp63; Tauroursodeoxycholic acid

2.2. Introduction

Alzheimer's disease (AD) is the most common human age-related, sporadic neurodegenerative disorder, and is characterized by a global cognitive decline. The major histopathological hallmarks of the disease are deposition of amyloid β (Aβ) peptide in neuritic plaques, formation of neurofibrillary tangles, and synaptic loss (Selkoe, 2001a). Aβ has been shown to play a critical role in the pathogenesis of AD. Among its two predominant forms, $A\beta_{1-42}$ possesses stronger aggregation and deposition propensity than $A\beta_{1-40}$ (Selkoe, 2001a). Previous studies have shown that Aβ-induced toxicity involves oxidative stress, inflammation, perturbation of calcium homeostasis, and most importantly apoptosis (Anderson et al., 1996; Estus et al., 1997; Loo et al., 1993; Selkoe, 2001a). In this respect, we have previously demonstrated that p53 participates in Aβ-induced apoptosis of PC12 neuronal cells through modulation of Bax expression (Ramalho et al., 2004). Furthermore, Aβ-induced apoptosis in rat primary cortical neurons was associated with translocation of pro-apoptotic Bax to the mitochondria, followed by cytochrome *c* (cyt *c*) release, caspase activation, and DNA and nuclear fragmentation (Solá et al., 2003a). Interestingly, a novel biological function of intracellular $A\beta_{1-42}$ has been suggested, where it acts as a transcription factor for the p53 promoter, enhancing p53-dependent neuronal apoptosis in AD (Ohyagi et al., 2005).

The role of apoptosis in Aβ-induced toxicity suggests that its modulation may slow the neurodegenerative process. Ursodeoxycholic acid (UDCA) and its taurine conjugate, tauroursodeoxycholic acid (TUDCA) are well characterized endogenous potent inhibitors of apoptosis in different cell types and their effects have expanded to several experimental models of neurological disorders, including AD (Ramalho et al., 2008). Several lines of evidence suggest that TUDCA modulates Aβ-induced neuronal death by inhibiting the mitochondrial machinery and enhancing survival signaling (Ramalho et al., 2006; Ramalho et al., 2004; Solá et al., 2006; Solá et al., 2003a). TUDCA is also a potent suppressor of Aβ-induced changes of lipid/protein fluidity and oxidative status in mitochondrial membranes (Rodrigues et al., 2001). Finally, TUDCA prevents an endoplasmic-reticulum-specific apoptotic pathway involved in Aβ neurotoxicity (Viana et al., 2011).

The p63 protein is a member of the p53 family that includes p53, p63 and p73. Alternative splicing and promoter usage results in multiple p63 isoforms with different biological activities. The p63 gene contains two promoters, leading to the

expression of two groups of proteins. Full-length proteins contain a transactivation (TA) domain (TAp63), whereas truncated proteins lack the TA domain (ΔNp63) (Yang et al., 1998). Additionally, both groups of proteins undergo carboxi-terminal alternative splicing giving rise to at least 6 different isoforms. TA isoforms act similarly to p53 and have the ability to transactivate p53 target genes and induce apoptosis. In contrast, ΔN isoforms have little transactivation activity and are thought to play a role in blocking transactivation of target genes of both p53 and TA isoforms (Yang et al., 2002). Therefore, ΔN isoforms function, at least in part, as naturally occurring dominant-inhibitory p53 family members. Both TA and ΔNp63 proteins are expressed in the cortex of embryonic, postnatal, and adult brains (Jacobs et al., 2005). Moreover, TAp63 has been shown to be required for growth factor-deprivation-mediated apoptosis of developing sympathetic neurons (Jacobs et al., 2005).

Given its key biological role, p63 is normally kept under tight regulatory control, mainly at the level of protein stability (Galli et al., 2010). Apoptotic doses of UV irradiation or genotoxic drugs promote phosphorylation-induced, proteasome-mediated degradation of the pro-survival isoform ΔNp63α (Chatterjee et al., 2008; Lazzari et al., 2011; Liefer et al., 2000; Papoutsaki et al., 2005; Westfall et al., 2005). Surprisingly, the mechanisms regulating p63 levels under cellular insults such as Aβ-mediated apoptosis have yet to be elucidated.

The c-Jun transcription factor, a component of the AP-1 family, is a critical regulator of both cell survival and death that responds to genotoxic stress. Importantly, c-Jun induction has been shown to occur after Aβ treatment *in vitro* (Estus et al., 1997; Iwasaki et al., 1996; Viana et al., 2010) and in AD brains (Anderson et al., 1994; Anderson et al., 1996), suggesting a possible requirement of this transcription factor for Aβ toxicity. In this respect, it was also reported that sympathetic neurons from c-Jun-deficient mice were resistant to $A\beta_{1-40}$ toxicity (Kihiko et al., 1999) and that extracellular application of aggregated Aβ induces neuronal apoptosis with c-Jun activation (Estus et al., 1997; Kihiko et al., 1999). Additionally, in AD patients, an increase in c-Jun immunoreactivity was reported in the brain (Anderson et al., 1996). These results suggest that c-Jun activation is a predominant feature of AD pathology and extracellular Aβ accumulation is a trigger of c-Jun activation. As c-Jun has been shown to regulate the abundance of TAp63

(Yao et al., 2010a), we hypothesized that c-Jun would have a key role in Aβ-mediated potential regulation of p63 stability.

The results presented here demonstrate that Aβ-induced apoptosis is associated with increased TAp63 and decreased ΔNp63 levels. Furthermore, our results strongly support the existence of a c-Jun-dependent mechanism regulating the abundance of anti-apoptotic ΔNp63 in the context of Aβ-induced stress. Importantly, TUDCA was efficient in partially modulating these effects.

2.3. Material and methods

2.3.1. Cell culture and treatments

Rat adrenal pheochromocytoma PC12 cells were grown in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated horse serum (Sigma-Aldrich), 5% fetal bovine serum (Invitrogen Corp., Carlsband, CA, USA) and 1% penicillin/streptomycin (Invitrogen Corp.), and maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were plated and differentiated in the presence of nerve growth factor (Promega Corp., Madison, WI, USA) for 6 days, as previously described (Viana et al., 2010). Cell density was either 1×10^5 cells/cm^2 for morphological assessment of apoptosis, or 4×10^5 cells/cm^2 for protein extraction, and transfection assays. PC12 neuronal-like cells were then preincubated in medium supplemented with 100 μM TUDCA (Sigma-Aldrich), or no addition (control), for 12 h, and then exposed to 25 μM of peptide active fragment $A\beta_{25–35}$ (Bachem AG, Bubendorf, Switzerland) for 2, 8, and 24 h. This was found to be the ideal concentration of soluble Aβ to achieve maximum cellular response with minimum exacerbated toxic effects in neuronal-like PC12 cells (Viana et al., 2010). In parallel experiments, cells were incubated with either 10 μM soluble $A\beta_{1-40}$ (Bachem AG), or 20 μM fibrillar $A\beta_{1-42}$ (Bachem AG) for 24 h (Viana et al., 2011). The predominant aggregates in these preparations are thought to be low N-oligomers, mainly monomeric to tetrameric, as fibrillogenesis usually requires longer incubation times and higher concentrations of the peptides [27]. Although high, the concentration of Aβ peptides used in this study are below the concentration of $A\beta_{1-42}$ found in the cerebrospinal fluid of AD patients [28, 29]. For the experiments with the MAPK and proteasome inhibitors, cells were pretreated with the JNK inhibitor SP600125 (10 μM), ERK inhibitor U0126 (50 μM), p38 inhibitor SB202190 (50 μM) (all from Sigma-Aldrich) or the proteasome inhibitor MG132 (5 μM; Calbiochem,

San Diego California**,** USA) for 1 h, in the presence or absence of $A\beta_{25–35}$ incubated for 24 h. Differentiated PC12 cells were also incubated for 6 h with genotoxic drugs doxorubicin (5 μM; Sigma-Aldrich) and cisplatin (20 μM; Sigma-Aldrich) as controls.

2.3.2. Primary culture of cortical neurons

Primary cultures of cortical neurons were prepared from embryonic (gestational day 18) CD1 mice and maintained in MEM Eagle's HBSS medium (Invitrogen). After dissociation with trypsin 0.25% (Invitrogen Corp.) and centrifugation, the cells were ressuspended in Neurobasal medium (Invitrogen Corp.), supplemented with 2% B-27 (Invitrogen), 0.5 mM glutamine (Invitrogen Corp.), 25 μM glutamic acid (Merck, Darmstadt, Germany), and 10 mg/mL gentamycin (Invitrogen Corp.). Neurons were plated onto 35 mm dishes coated with poly-D-lysine (0.05 mg/ml, Sigma-Aldrich) at a density of 1.0×10^6cells/cm^2. The cultures were kept at 37ºC in a humidified incubator with a 5% CO_2 atmosphere. The medium was replaced half on day 4 with Neurobasal medium without glutamic acid. TUDCA and $A\beta_{25–35}$ treatment was performed as described before for PC12 cells, starting at day 4 in culture.

2.3.3. Measurement of cell death

Apoptotic nuclei were detected by Hoechst labeling after cell fixation with 4% formaldehyde in phosphate buffer saline (PBS), for 10 min at room temperature. Following incubation with Hoechst dye 33258 (Sigma-Aldrich) at 5 μg/mL in PBS for 5 min, and PBS washes, slides were mounted with Fluoromount-G (Beckman Coulter, Inc, Fullerton, CA, USA) and fluorescence visualized with an Axioskop fluorescence microscope (Carl Zeiss GmbH, Hamburg, Germany). Fluorescent nuclei were scored and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed non-condensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were independently identified by condensed and fragmented chromatin contiguous to the nuclear membrane, as well as nuclear fragmentation and presence of apoptotic bodies. Three random microscopic fields per sample of ~250 nuclei were counted and mean values expressed as the percentage of apoptotic nuclei.

2.3.4. Plasmids, siRNAs and transfections

For the identification of the endogenous p63 isoforms, PC12 cells were transfected using Lipofectamine 2000 (Invitrogen Corp.) with 8 μg pcDNA3.1-ΔNp63α, -ΔNp63γ, TAp63α or TAp63γ expression plasmids (kind gift from Dr. Kurt Engeland, University of Leipzig). In subsequent experiments, 4 μg pSG5-c-Jun expression plasmid (kind gift from Dr. Imakawa, University of Tokyo), 2 μg pcDNA3.1-ΔNp63γ, or 100 nM c-Jun siRNA (Dharmacon, Waltham, MA, USA) were used. As controls, cells were transfected with the corresponding empty vector plasmids, or a control siRNA (Dharmacon) containing a scrambled sequence that does not lead to the specific degradation of any known cellular mRNA. Total amount of transfected nucleic acids was equalized with appropriate amounts of empty vector in all cases. Cells were collected 48 h after transfection for immunoblot analyses. In experiments with the protein synthesis inhibitor cycloheximide (CHX), cells were transfected with pSG5-c-Jun and pcDNA3.1-ΔNp63γ plasmids 48 h prior to addition of CHX (50 μg⁄mL; Sigma-Aldrich), and maintained for the indicated time points. In addition, c-Jun siRNA was transfected 48 h prior to addition of $A\beta_{25–35}$ and maintained throughout the 24 h of Aβ incubation. Floating and attached cells were harvested, and total protein extracts were prepared for Western blot analysis.

3.3.5. Western blot

Protein levels of TAp63, ΔNp63 and c-Jun in PC12 neuronal-like cells and primary cortical neurons were determined by Western blot analysis. Briefly, 100 μg of total proteins were separated by 8% sodium dodecyl-polyacrylamide gel electrophoresis. After electrophoretic transfer onto nitrocellulose membranes, and blocking with a 5% milk solution, membranes were incubated overnight at 4ºC with a primary mouse monoclonal antibody reactive to p63 (4A4; Santa Cruz Biotechnology, Santa Cruz, CA, USA), which detects both TAp63 and ΔNp63 isoforms, and a primary rabbit monoclonal antibody reactive to c-Jun (60A8; Cell Signaling Technology, Inc., Danvers, MA, USA). Finally, secondary goat anti-mouse or anti-rabbit IgG antibody conjugated with horseradish peroxidase (BioRad Laboratories, Hercules, CA, USA) was added for 3 h at room temperature. The membranes were processed for protein detection using the SuperSignal substrate (Pierce Biotechnology, Rockford, IL, USA). β-Actin (AC-15; Sigma-Aldrich) was used as

loading control. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories), according to the manufacturer's specifications.

2.3.6. Statistical analysis

The relative intensities of protein bands were analyzed using the QuantityOne Version 4.6 densitometric analysis program (Bio-Rad Laboratories) and normalized to the respective loading controls. Statistical analysis was performed using GraphPad InStat version 3.00 (Graph-Pad Software) for the analysis of variance and Bonferroni's multiple comparison tests. Values of $p < 0.05$ were considered significant.

2.4. Results

2.4.1. Aβ-induced cell stress is associated with decreased ΔNp63 and increased c-Jun protein levels

Expression of p63 isoforms was first confirmed in PC12 neuronal-like cells after overexpression of either TAp63α, TAp63γ, ΔNp63α, or ΔNp63γ. Endogenous TAp63γ (~ 57 kDa) and ΔNp63γ (~ 54 kDa) were detected from a group of at least six different isoforms encoded by p63 (Fig. 2.1A). In addition, endogenous expression of ΔNp63γ was more than 5-fold greater than that of TAp63γ.

Genotoxic signals are known to regulate p63 (Kirschner et al., 2008; Liefer et al., 2000; Papoutsaki et al., 2005; Yao et al., 2010a). In fact, exposure of PC12 neuronal-like cells to classic genotoxic insults, such as doxorubicin and cisplatin resulted in increased TAp63 levels, concurrent with the reduction of ΔNp63 abundance (Fig. 2.1B). To determine whether p63 levels are also controlled by Aβ-induced stress, we performed a time-course analysis of $A\beta_{25-35}$ treatment in PC12 neuronal-like cells. Our results show that Aβ treatment resulted in stabilization of TAp63 and, most importantly, reduction of ΔNp63 (Fig. 2.2A). These effects were more evident after 24 h of Aβ treatment. c-Jun, a key regulator of stress-mediated cell death has been shown to modulate the abundance of TAp63 (Yao et al., 2010a). We therefore investigated if c-Jun would have any role in the Aβ-mediated regulation of p63 levels. Time-course analysis of $A\beta_{25-35}$ treatment revealed that c-Jun protein levels increased with time, peaking at 24 h after Aβ treatment (Fig. 2.2A). Similarly, exposure of PC12 neuronal-like cells to classic genotoxic insults resulted in c-Jun upregulation (Fig. 2.1B). Finally, to confirm Aβ role on the regulation of p63 levels,

PC12 cells were incubated with the full-length soluble $A\beta_{1-40}$ and fibrillar $A\beta_{1-42}$ peptides. As predicted, similarly to $A\beta_{25-35}$, both $A\beta_{1-40}$ and $A\beta_{1-42}$ decreased ΔNp63 levels while increasing TAp63 and c-Jun levels (Fig. 2.2B). These results suggest the existence of an inverse relationship between ΔNp63 and c-Jun levels following Aβ treatment.

To investigate if Aβ-mediated reduction of ΔNp63 protein levels is influenced by the mitogen activated protein kinase (MAPK) pathway, PC12 neuronal-like cells were treated with MAPK inhibitors for 1 h before Aβ incubation, and p63 levels were examined by Western blot analysis. Treatment with the JNK inhibitor SP600125, ERK inhibitor PD98059 and p38 inhibitor SB202190 rescued the reduction of ΔNp63 levels observed after Aβ treatment (Fig. 2.2C).

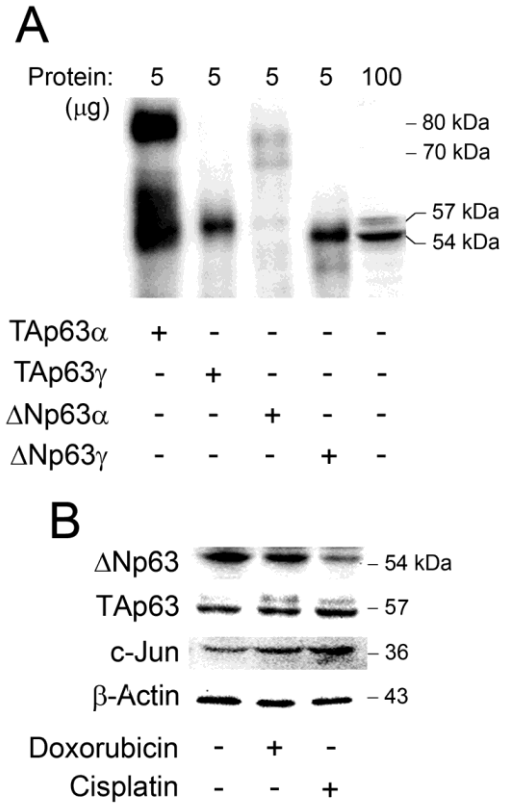


Figure 2.1. Expression of endogenous p63 isoforms and genotoxic stress-induced regulation of p63 and c-Jun protein levels in PC12 neuronal-like cells. *A) Detection of p63 isoforms in cells transfected with the overexpression plasmid TAp63α, TAp63γ, ΔNp63α, or ΔNp63γ for 48 h, compared with untransfected cells. Total proteins were extracted from transfected and untransfected cells and used for Western blot analysis of 5 and 100 μg, respectively. p63 protein isoforms were identified using the pan-p63 monoclonal antibody 4A4, as described in Materials and Methods. B) Protein levels of ΔNp63, TAp63 and c-Jun in cells incubated with 5 μM doxorubicin or 20 μM cisplatin for 6 h, or no addition (control).*

β-actin was used as loading control. Immunoblots are representative of at least three different experiments.

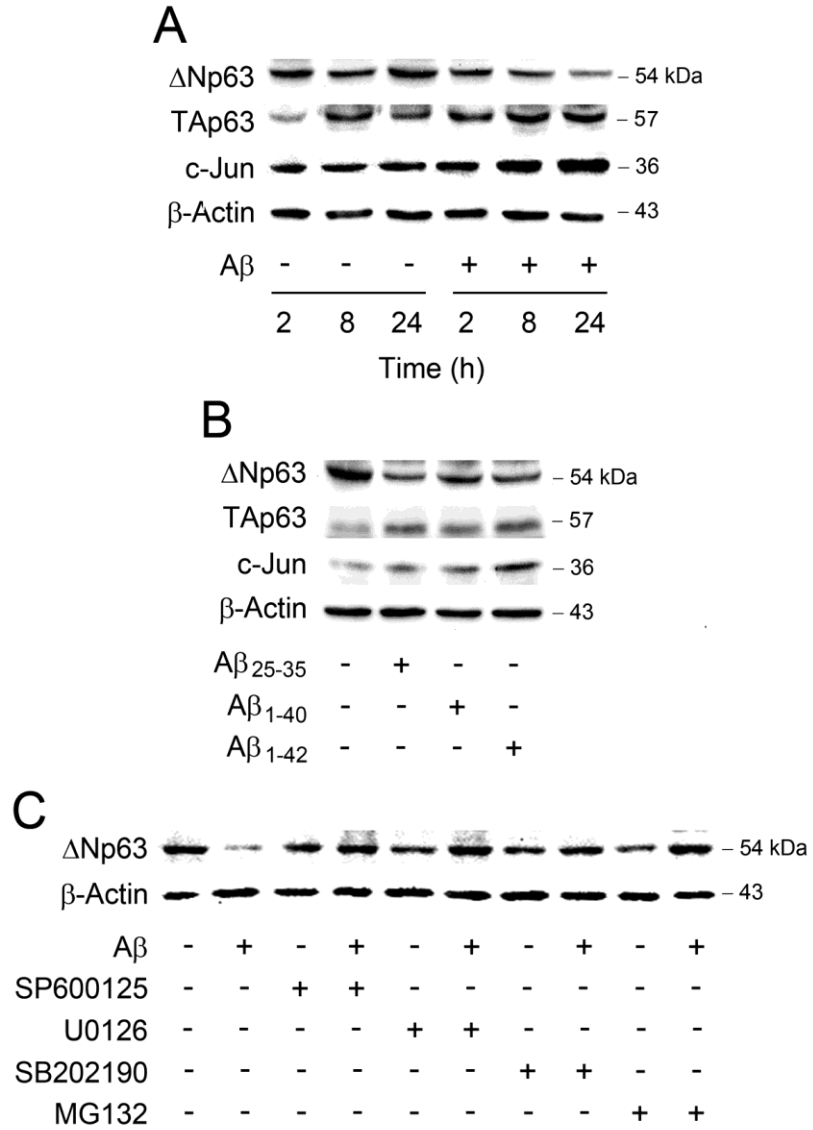


Figure 2.2. Aβ-induced cell stress is associated with decreased ΔNp63 and increased TAp63 and c-Jun protein levels in PC12 neuronal-like cells. *Cells were incubated with 25 μM soluble $Aβ_{25–35}$ or no addition (control). Total proteins were extracted for Western blot analysis as described in Materials and Methods. β-actin was used as loading control. A) Protein levels of ΔNp63, TAp63 and c-Jun in cells incubated with $Aβ_{25–35}$ for 2, 8, and 24 h. B) Protein levels of ΔNp63, TAp63 and c-Jun in cells incubated with $Aβ_{25–35}$, 10 μM soluble $Aβ_{1-40}$, or 20 μM fibrillar $Aβ_{1-42}$ for 24 h. C) Protein levels of ΔNp63 in cells incubated with the JNK inhibitor (SP600125), ERK inhibitor (U0126), p38 inhibitor (SB202190) or proteasome inhibitor MG132 for 1 h, in the presence or absence of $Aβ_{25–35}$ incubated for 24 h. β-actin was used as loading control. Immunoblots are representative of at least three different experiments.*

We also investigated the effect of directly inhibiting the proteasome by treating cells with the proteasome inhibitor MG132.

Treatment with the proteasome inhibitor abolished the Aβ-dependent reduction of ΔNp63 levels (Fig. 2.2C), indicating that ΔNp63 degradation occurs via the proteasome. These results suggest that the decrease in ΔNp63 protein levels in cells exposed to Aβ is MAPK pathway- and proteasome-dependent.

2.4.2. TUDCA modulates Aβ-induced changes in ΔNp63 and c-Jun protein levels

It has been previously shown that soluble $A\beta_{25-35}$ fragment induces apoptosis in PC12 neuronal-like cells and cortical neurons, which is abrogated by TUDCA pretreatment (Ramalho et al., 2004; Solá et al., 2003a; Viana et al., 2010). In this study, rat PC12 neuronal-like cells and primary cortical neurons were incubated with $A\beta_{25-35}$, with or without pre-treatment with the anti-apoptotic bile acid TUDCA, and assessed for cell death as well as ΔNp63 and c-Jun protein levels. Incubation with $A\beta_{25-35}$ for 24 h induced apoptosis in ~ 13 and 40% of PC12 cells and cortical neurons, respectively ($p < 0.01$) (Fig. 2.3A). Pre-treatment with TUDCA markedly reduced Aβ-induced apoptosis ($p < 0.05$). Notably, TUDCA prevented Aβ-induced ΔNp63 degradation in both PC12 cells and cortical neurons, possibly by restoring c-Jun protein levels to almost control values ($p < 0.01$ in PC12 cells, and $p < 0.05$ in cortical neurons). These results suggest that inhibition of ΔNp63 degradation through modulation of c-Jun levels may also account for TUDCA neuroprotective mechanism.

2.4.3. c-Jun is required for ΔNp63 degradation after Aβ treatment

To clarify whether c-Jun could directly lead to ΔNp63 degradation, c-Jun was overexpressed in PC12 cells. c-Jun overexpression led to a reduction of endogenous ΔNp63, compared with cells transfected with the empty vector pSG5 (Fig. 2.4A). Additionally, transient co-expression of c-Jun and ΔNp63 caused a marked decrease of exogenous ΔNp63 ($p < 0.01$) (Fig. 2.4B), even with high level expression reached after transfection, suggesting that c-Jun may be a potent negative regulator of ΔNp63 abundance.

To further investigate the role of c-Jun in regulating ΔNp63 abundance, we then examined whether ΔNp63 protein levels were altered by silencing endogenous c-Jun. Immunobloting analysis revealed that knockdown of c-Jun by specific c-Jun siRNA abrogated the reduction of ΔNp63 levels following Aβ stress, when compared with control siRNA-transfected cells ($p < 0.05$) (Fig. 2.5A).

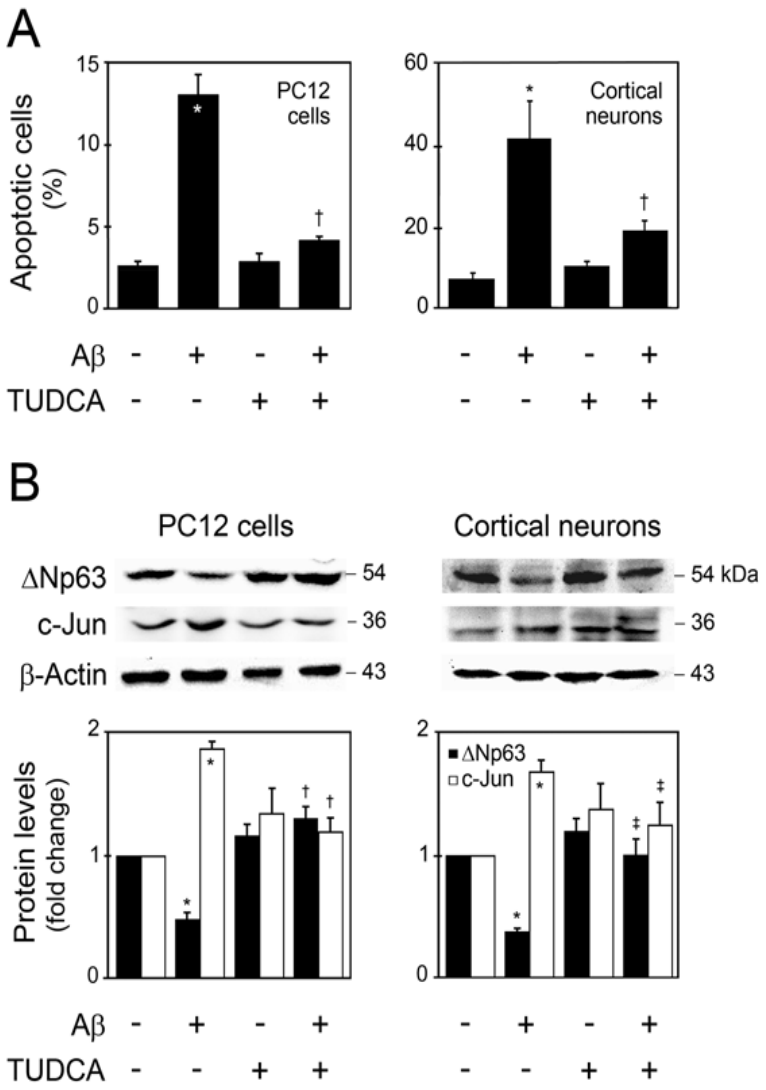


Figure 2.3. TUDCA modulates Aβ-induced apoptotic changes in ΔNp63 and c-Jun protein levels in PC12 neuronal-like cells and cortical neurons. *Cells were incubated for 24 h with 25 μM soluble $A\beta_{25-35}$, or no addition (control), in the presence or absence of 100 μM TUDCA. In co-incubation experiments, TUDCA was added 12 h prior to incubation with Aβ. Cells were fixed and stained for microscopic assessment of apoptosis and total proteins were extracted for Western blot analysis as described in Materials and Methods. A) Percentage of apoptosis after Hoechst staining in cells exposed to Aβ with or without TUDCA pretreatment. Results are expressed as mean ± SEM for at least three different experiments. *p < 0.01 from control and †p < 0.05 from Aβ alone. B) Protein levels of ΔNp63 and c-Jun in cells exposed to Aβ with or without TUDCA pre-treatment. Representative immunoblot of ΔNp63 and c-Jun (top) and respective protein levels (bottom). Results were normalized to endogenous β-Actin protein levels and expressed as mean ± SEM for at least four different experiments. *p < 0.01 from control; †p < 0.01 and ‡p < 0.05 from Aβ alone.*

These results strongly suggest that Aβ-mediated ΔNp63 degradation occurs in a c-Jun-dependent manner. Furthermore, knockdown of endogenous c-Jun in cells transfected with ΔNp63 plasmid resulted in increased expression of exogenous

ΔNp63 ($p < 0.01$) (Fig. 2.5B). These results further corroborate the hypothesis that c-Jun plays an important role in regulating ΔNp63 abundance.

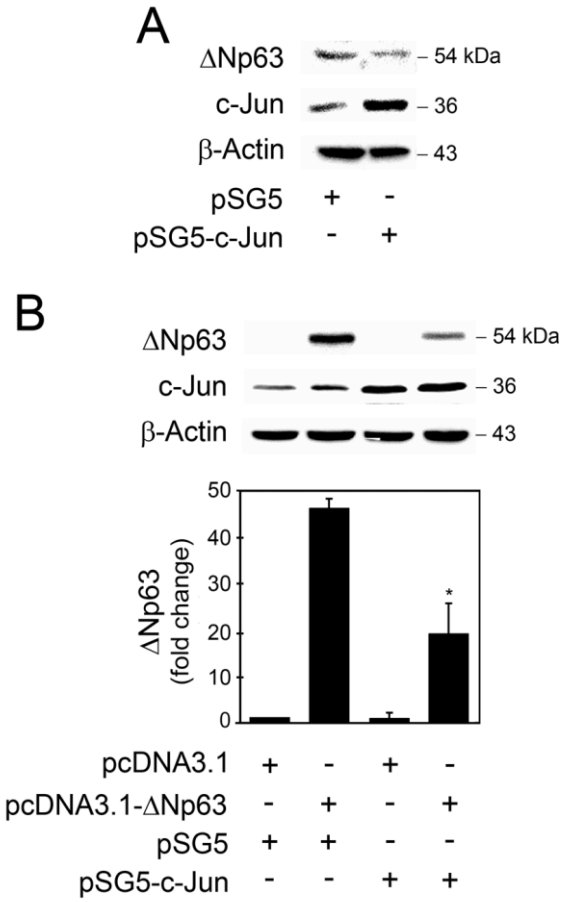


Figure 2.4. Overexpression of c-Jun downregulates both endogenous and exogenous levels of ΔNp63 in PC12 neuronal-like cells. *Cells were transfected with c-Jun overexpression plasmid in the presence or absence of ΔNp63γ overexpression plasmid. After 48 h of transfection, total proteins were extracted for Western blot analysis as described in Materials and Methods. A) Protein levels of ΔNp63 and c-Jun in cells transfected with c-Jun overexpression plasmid (pSG5-c-Jun) or its corresponding empty vector (pSG5). B) Protein levels of ΔNp63 and c-Jun in cells co-transfected with c-Jun overexpression plasmid (pSG5-c-Jun), or its corresponding empty vector (pSG5), and ΔNp63γ overexpression plasmid (pcDNA3.1-ΔNp63), or its corresponding empty vector (pcDNA3.1). Representative immunoblot of ΔNp63 and c-Jun (top) and respective protein levels (bottom). Results were normalized to endogenous β-Actin protein levels and expressed as mean ± SEM for at least four different experiments. *$p < 0.01$ from ΔNp63 overexpressing cells.*

Finally, to better understand c-Jun contribution to ΔNp63 degradation, we investigated ΔNp63 half-life in the presence of c-Jun. For this purpose, PC12 cells transfected with ΔNp63 alone or together with c-Jun were treated with the protein synthesis inhibitor CHX.

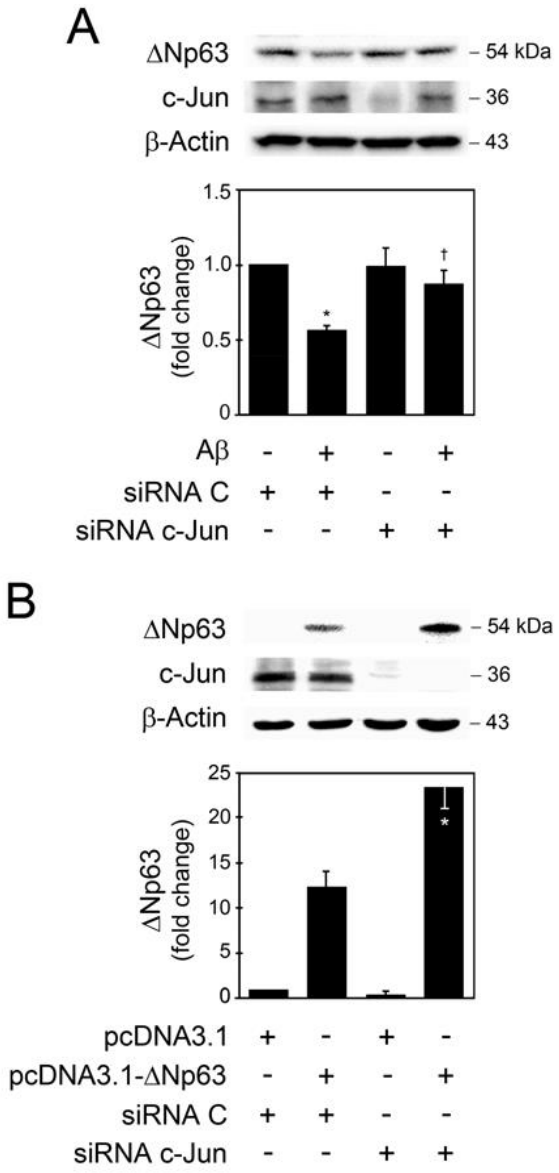


Figure 2.5. Silencing of endogenous c-Jun abrogates Aβ-induced ΔNp63 degradation and increases exogenous ΔNp63 levels in PC12 neuronal-like cells. *Cells were transfected with 100 nM of either c-Jun siRNA or control siRNA (siRNA C) for 48 h. A) After 48 h of siRNA transfections, cells were challenged with 25 μM soluble $Aβ_{25–35}$, or no addition (control), for 24 h. *p < 0.01 from control cells transfected with siRNA control (siRNA C); †p < 0.05 from Aβ-incubated cells transfected with siRNA control. B) ΔNp63γ overexpression plasmid (pcDNA3.1-ΔNp63) and its corresponding empty vector (pcDNA3.1) were co-transfected with c-Jun and control siRNAs. Total proteins were extracted for Western blot analysis as described in Materials and Methods. Representative immunoblots of ΔNp63 and c-Jun (top) and respective protein levels (bottom). Results were normalized to endogenous β-Actin protein levels and expressed as mean ± SEM for at least three different experiments. *p < 0.01 from ΔNp63 overexpressing cells transfected with siRNA control (siRNA C).*

Results demonstrate that the highly stable ΔNp63 half-life was reduced to less than 8 h in the presence of c-Jun ($p < 0.01$) (Fig. 2.6), confirming that c-Jun indeed regulates the stability of ΔNp63.

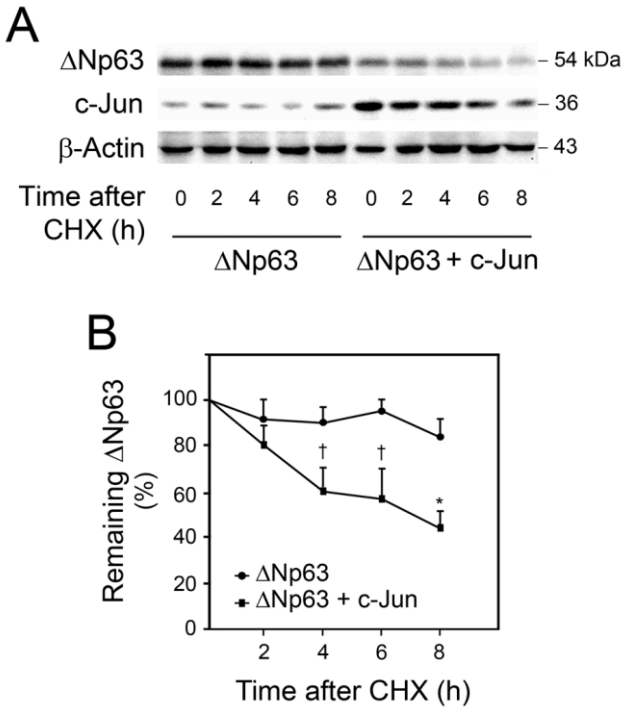


Figure 2.6. c-Jun decreases ΔNp63 half-life in PC12 neuronal-like cells. *Cells transfected with ΔNp63γ alone (ΔNp63) or with c-Jun (ΔNp63 + c-Jun) were untreated (0 h) or treated with 50 μg/mL CHX for 2, 4, 6, and 8 h. At the indicated time points, total proteins were extracted for immunoblot analysis as described in Materials and Methods. A) Representative immunoblots of ΔNp63 and c-Jun after CHX treatment. B) Percentage of remaining ΔNp63 after CHX treatment. Results were normalized to endogenous β-Actin protein levels and expressed as mean ± SEM for at least four different experiments. *p < 0.01 and †p < 0.05 from ΔNp63 overexpressing cells.*

2.5. Discussion

Aβ-induced toxicity is a multifactorial process that ultimately leads to apoptotic neuronal death. In the present study, we show that Aβ induces an antagonistic effect in the regulation of p63 isoform abundance in PC12 neuronal-like cells, with upregulation of the pro-apoptotic isoform TAp63γ and downregulation of the anti-apoptotic isoform ΔNp63γ. A similar effect was observed by incubation of PC12 neuronal-like cells with the genotoxic drugs doxorubicin and cisplatin. Previous studies have already shown that treatment of cells with various DNA-damaging agents resulted in increased expression of TAp63 isoforms (Hershkovitz Rokah et al., 2010; Kirschner et al., 2008; Sayan et al., 2007a; Yao et al., 2010a). A downregulation of ΔNp63 in response to UV irradiation or genotoxic drugs was only reported for the α isoform (Chatterjee et al., 2008; Lazzari et al., 2011; Liefer et al., 2000; Papoutsaki et al., 2005; Westfall et al., 2005). Further, we show that Aβ

induces apoptosis in rat cortical neurons by regulating the abundance of both antagonistic isoforms of p63, TAp63γ and ΔNp63γ.

The many isoforms generated from the p63 gene provide the molecular flexibility to play very different, and even functionally antagonistic roles, depending on the cellular context. Indeed, TAp63 isoforms are often upregulated after exposure to cellular insults, contributing to cell death (Hershkovitz Rokah et al., 2010; Kirschner et al., 2008; Sayan et al., 2007a; Yao et al., 2010a), as TAp63 can activate both death receptor- and mitochondrial-mediated apoptotic pathways (Gressner et al., 2005). Similarly, stress signals trigger the reduction of ΔNp63 levels (Chatterjee et al., 2008; Lazzari et al., 2011; Liefer et al., 2000; Papoutsaki et al., 2005; Westfall et al., 2005), which will allow the manifestation of apoptosis. The balance between these two major p63 isoforms dictates cell fate (Vanbokhoven et al., 2011). Because both isoforms have profoundly distinct biological effects, it is not surprising that they are differently regulated by Aβ-induced stress signaling. As shown here, exposure of both PC12 neuronal-like cells and cortical neurons to Aβ active fragment $A\beta_{25–35}$ resulted in apoptotic cell death. In addition, $A\beta_{25–35}$ and the full-length Aβ peptides, $A\beta_{1-40}$ and $A\beta_{1-42,}$ increased TAp63 and decreased ΔNp63 levels. Importantly, the anti-apoptotic bile acid TUDCA strongly modulated Aβ-induced apoptosis while preventing the changes in p63 levels, further suggesting that the reduction of ΔNp63 levels is important for apoptosis to occur.

c-Jun transcriptionally activates the TAp63 promotor (Yao et al., 2010a), possibly contributing to some forms of c-Jun-mediated cell death. Furthermore, c-Jun is increased after Aβ exposure and in AD brains (Anderson et al., 1994; Anderson et al., 1996; Estus et al., 1997) suggesting a role for c-Jun in Aβ-induced toxicity. We have recently shown that JNK is the proximal stress sensor for Aβ-induced toxicity, and that it translocates to the nucleus and activates caspase-2 in PC12 neuronal cells (Viana et al., 2010). Caspase-2 in turn induces cleavage of golgin-160, suggesting a unique transduction mechanism of Aβ apoptotic signaling through the Golgi complex. This led us to hypothesize that c-Jun could be responsible for Aβ-dependent decrease in ΔNp63 protein levels. Here, we demonstrate that ΔNp63 reduction was associated with increased c-Jun protein levels after Aβ treatment of PC12 neuronal-like cells and cortical neurons. Moreover, TUDCA rescued Aβ-induced decrease of ΔNp63 levels and prevented the increase in c-Jun levels. Importantly, c-Jun overexpression led to ΔNp63 degradation, whereas knockdown of c-Jun by specific c-Jun siRNA abrogated

the reduction of ΔNp63 following Aβ stress. Finally, the highly stable ΔNp63 half-life was reduced in the presence of c-Jun. These results clearly show that Aβ-mediated ΔNp63 degradation occurs in a c-Jun-dependent manner, and highlight the existence of distinct pathways that have probably evolved for the reciprocal regulation of the abundance of functionally different but highly homologous proteins TAp63 and ΔNp63 under Aβ stress.

c-Jun is one of the components of the transcription factor AP-1 and its expression is regulated by a variety of signals in different cell types (Hess et al., 2004). Despite the extensive knowledge regarding c-Jun activation, downstream cell signaling events remain unclear in Aβ-induced cell stress. Here we demonstrate that ΔNp63γ is a downstream target of c-Jun in Aβ-induced apoptosis. In fact, after depleting cells of c-Jun by specific siRNA, Aβ failed to decrease ΔNp63 protein levels. c-Jun is a major target of the MAPK pathway as it contributes to the cellular stress response and is believed to mediate neuronal apoptosis (Behrens et al., 1999; Suganuma et al., 2010). We therefore explored whether Aβ-mediated ΔNp63γ reduction was influenced by the MAPK pathway. Our results show that in the presence of the JNK, ERK or p38 inhibitors, Aβ no longer resulted in decreased ΔNp63. The same was true for the incubation with the proteasome inhibitor, suggesting that the decrease in ΔNp63 protein levels in cells exposed to Aβ was MAPK pathway- and proteasome-dependent. In the past few years, some ΔNp63α-modifying enzymes have been identified and reported to regulate ΔNp63α stability (Bakkers et al., 2005). These proteins bind to sites in the C-terminal region of ΔNp63α, which are absent in the shorter and more stable ΔNp63γ isoform. Future studies are needed to clarify the exact mechanism by which c-Jun targets ΔNp63γ isoform to proteasomal-dependent degradation. Despite the fact that Aβ may inhibit the proteasome [39, 40], our results are in accordance with others demonstrating that important mediators of AD are degraded in a proteasome-dependent fashion [41, 42], thus contributing to AD pathogenesis.

In conclusion, this study clearly shows that Aβ exposure differently regulates TAp63 and ΔNp63 isoforms to allow the manifestation of apoptosis. This suggests that the selective regulation of the two antagonistic isoforms of p63 is crucial in maintaining the homeostatic balance between life and death of a cell that is undergoing Aβ-induced cell stress. The present study also convincingly shows that c-Jun is required for Aβ-mediated degradation of ΔNp63, which occurs via MAPK

pathway and proteasome activation. Thus, c-Jun appears to be a stress sensor for Aβ-induced apoptosis, which in turn is strongly modulated by TUDCA. These results provide novel insights into the specific cellular mechanism of Aβ-induced neuronal apoptosis and suggest novel targets for potential therapeutic intervention.

Acknowledgments

MBF and AFN are recipients of PhD (SFRH/BD/43959/2008) and postdoctoral (SFRH/BPD/34603/2007) fellowships, respectively, from Fundação para a Ciência e a Tecnologia, Portugal. The authors have no conflict of interest to declare.

TAp63γ Demethylation Regulates Protein Stability and Cellular Distribution during Neural Stem Cell Differentiation

Maria B. Fonseca[1], Ana F. Nunes[1], Ana L. Morgado[1], Susana Solá[1,2], Cecília M. P. Rodrigues[1,2]

[1]Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL), and [2]Department of Biochemistry and Human Biology, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal

PLoS ONE 2012; 7 (12): e52417

Reprinted from *PLoS ONE* vol 7, Issue 12, Maria B. Fonseca, Ana F. Nunes, Ana L. Morgado Susana Solá, Cecília M. P. Rodrigues. TAp63γ Demethylation regulates protein stability and cellular distribution during neural stem cell differentiation, page e52417, Copyright 2012, Fonseca *et al.* All rights reserved.

3.1. Abstract

p63 is a close relative of the p53 tumor suppressor and transcription factor that modulates cell fate. The full-length isoform of p63, containing a transactivation (TA) domain (TAp63) is an essential proapoptotic protein in neural development. The role of p63 in epithelial development is also well established; however, its precise function during neural differentiation remains largely controversial. Recently, it has been demonstrated that several conserved elements of apoptosis are also integral components of cellular differentiation; p53 directly interacts with key regulators of neurogenesis. The aim of this study was to evaluate the role of p63 during mouse neural stem cell (NSC) differentiation and test whether the histone H3 lysine 27-specific demethylase JMJD3 interacts with p63 to redirect NSCs to neurogenesis. Our results showed that JMJD3 and TAp63γ are coordinately regulated to establish neural-specific gene expression programs in NSCs undergoing differentiation. JMJD3 overexpression increased TAp63γ levels in a demethylase activity-dependent manner. Importantly, overexpression of TAp63γ increased β-III tubulin whereas downregulation of TAp63γ by specific p63 siRNA decreased β-III tubulin. Immunoprecipitation assays demonstrated direct interaction between TAp63γ and JMJD3, and modulation of TAp63γ methylation status by JMJD3-demethylase activity. Importantly, the demethylase activity of JMJD3 influenced TAp63γ protein stabilization and cellular distribution, as well as TAp63γ-regulated neurogenesis. These findings clarify the role of p63 in adult neural progenitor cells and reveal TAp63γ as a direct target for JMJD3-mediated neuronal commitment.

Keywords: Differentiation; JMJD3; Neural stem cells; p63 post-translation modifications

3.2. Introduction

The transcription factor p63, member of the p53 family, can be expressed as a full-length isoform containing a transactivation (TA) domain, termed TAp63, or as a truncated isoform that lacks the TA domain, termed ΔNp63. This isoform functions, at least in part, as naturally occurring dominant-inhibitor of full-length p53 family members. Additionally, both TAp63 and ΔNp63 undergo C-terminal alternative splicing giving rise to at least 6 different isoforms, which along with post-translational modifications (PTM), accounts for molecular and regulatory complexity (Fonseca et al., 2012; Nicotera and Melino, 2005; Yang et al., 1998). Full-length p53 family members show structural and functional similarities, including a comparable domain structure of an N-terminal TA domain, an invariant DNA-binding domain, and an oligomerization domain (Courtois et al., 2004). p63 has been well-established as an important regulator of cell survival and cell death in the nervous system, primarily by acting through the apoptosis machinery (Fonseca et al., 2012; Jacobs et al., 2005; Nicotera and Melino, 2005). In this respect, we have shown that both TA and ΔNp63 isoforms are involved in molecular mechanisms associated with Alzheimer's disease (Fonseca et al., 2012).

Recently, it has been suggested that cell death-relevant proteins, especially those involved in the core of the executing apoptosis machinery, may play a dual function in differentiation and cell death (Fernando and Megeney, 2007). Restrictive activation and careful regulation will assure differentiation efficiency and thus avoid cell loss. In this regard, we have previously demonstrated the involvement of specific apoptosis-related microRNAs and proteins, including p53, caspases and calpains, in mouse neural stem cell (NSC) differentiation, by mechanisms that do not result in cell death (Aranha et al., 2011; Aranha et al., 2010; Aranha et al., 2009; Santos et al., 2012b; Solá et al., 2011b). Several studies have shown that inactivation of p53 sustains the undifferentiated state of neural precursor cells (Armesilla-Diaz et al., 2009; Meletis et al., 2006; Zheng et al., 2008), and that p53 is required for both neurite outgrowth and axonal regeneration in mice primary neurons (Di Giovanni et al., 2006). We have further dissected the role of p53 during NSC differentiation by showing that this proapoptotic protein cross-talks with key regulators of neurogenesis, such as the histone H3 lysine 27-specific demethylase JMJD3 (Solá et al., 2011b). In fact, PTM of apoptosis-related proteins may redirect stem cells to differentiation, as

an alternative to cell death, and establish tissue-specific programs of gene expression throughout differentiation. A strong interplay between p53, p63 and p73 isoforms has been recently demonstrated, which orchestrates cell fate decisions (Jost et al., 1997; Marcel et al., 2012). In addition, similar to p53, TAp63 can oligomerize, bind to DNA, transactivate p53 target genes, and induce cell cycle arrest and apoptosis (Fonseca et al., 2012; Jost et al., 1997; Yang et al., 1998).

The pivotal role of p63 during epithelial development has been well recognized. Mice lacking all p63 isoforms die at birth and show severe developmental abnormalities, including limb truncation, and defects in the epidermis and its appendages (Yang et al., 1999). However, although TAp63 critical function in sympathetic neuronal development is firmly established (Jacobs et al., 2005), two independent studies have recently reported that p63 is not essential for central nervous system development (Hernández-Acosta et al., 2011; Holembowski et al., 2011). Nevertheless, in contrast to p63-restricted expression observed during mice and human brain development, high levels of p63 protein and mRNA were observed in adult human cerebral cortex and hippocampus (Hernández-Acosta et al., 2011), suggesting a role for p63 in the adult brain. Furthermore, in postnatal mice, p63 was shown to be expressed in neurogenic niches, such as the subventricular zone of the lateral ventricle (Hernández-Acosta et al., 2011). Together, this evidence supports the idea that p63 may have a key role during adult neurogenesis.

Here, we investigated p63 involvement during adult mouse NSC differentiation and explored the potential cross-talk between p63 and JMJD3 in this cellular context. Our results demonstrated that TAp63γ and JMJD3 are coordinately regulated during NSC differentiation to establish a neuronal-specific gene expression program. In addition, TAp63γ subcellular distribution and neurogenic functions may be regulated by direct JMJD3-dependent demethylation of TAp63γ.

3.3. Material and methods

3.3.1. Ethics statement

The mouse NSC line used in this study was obtained from Dr. Smith's Laboratory, University of Cambridge, Cambridge, UK (Pratt et al., 2000; Silva et al., 2006; Solá et al., 2011b), and provided by Dr. Henrique, University of Lisbon, Lisbon, Portugal. The Animal Ethical Committee at the Faculty of Pharmacy, University of Lisbon, Portugal waived the need for approval.

3.3.2. Cell culture and differentiation

A mouse NSC line was derived from day 14.5 post-coitum mouse fetal forebrain (Pratt et al., 2000; Silva et al., 2006; Solá et al., 2011b). This cell line was established using a method that produces pure cultures of adherent NSCs, which continuously expand by symmetrical division, and are capable of tripotential differentiation (Conti et al., 2005; Glaser et al., 2007; Pollard et al., 2006). Cells were grown in monolayer as previously described (Santos et al., 2012b; Spiliotopoulos et al., 2009) and routinely maintained in undifferentiation medium, Euromed-N medium (EuroClone S.p.A., Pavia, Italy), supplemented with 1% N-2 supplement (Invitrogen Corp., Grand Island, NY), 20 ng/mL epidermal growth factor (EGF; PeproTech EC, London, UK), 20 ng/mL basic fibroblast growth factor (bFGF; PeproTech EC) and 1% penicillin-streptomycin (Invitrogen Corp.), in uncoated tissue culture plastic flasks at 37°C in a humidified atmosphere of 5% CO_2. Medium was changed every 3 days and cells collected with accutase (Sigma-Aldrich Co., St. Louis, MO) when confluent. Differentiation of NSCs was performed by first platting cells in undifferentiation medium onto uncoated tissue culture plastic dishes at 1 x 10^6 cells/cm^2 for 24 h, and changing the culture medium to differentiation medium, Euromed-N medium supplemented with 10 ng/mL bFGF, 0.5% N-2 supplement, 1% B27 supplement and 1% penicillin-streptomycin. Cells were collected before medium change (time 0), or cultured for additional 6, 12, 24 or 48 h, and then fixed for immunocytochemistry analysis or collected for protein extraction.

3.3.3. siRNAs and plasmid transfections

The NSC cell line was transfected with Flag-JMJD3 or Flag-JMJD3 mutant overexpression plasmids to amplify JMJD3 expression. The expression plasmids were kindly provided by Dr. Kristen Jepsen (University of California, San Diego, USA). The Flag-JMJD3 construct was cloned by inserting full-length mouse JMJD3 cDNA in frame into p3xFLAG CMV-10 (Sigma-Aldrich Co.) vector within HindIII and BamHI sites. The Flag-JMJD3 mutant was generated by removing the carboxy-terminus 410 amino acids, which include the jumonji C domain. Briefly, NSCs were first cultured in uncoated dishes in undifferentiation medium without penicillin-streptomycin. Twenty-four hours after plating, cells were transfected using Lipofectamine 2000 (Invitrogen Corp.), according to the manufacturer's instructions, the medium was changed to differentiation medium, and cells were cultured for

additional 24 h. The cDNAs for TAp63γ were ligated as BamHI/XhoI fragments into the pcDNA3.1/His C vector (Invitrogen Corp.). Mutant TAp63γ carries a R304H exchange to yield a mutation in the DNA binding domain, as previously described (Dietz et al., 2002). Briefly, the mutant expression vector includes a His at the equivalent Arg position, which is transcriptionally impaired. As a control (mock), cells were incubated with the transfection agent at the same concentration and time, in the absence of any plasmid. Attached cells were either harvested for immunobloting or fixed for immunocytochemistry and apoptosis assays using Hoechst staining. p63 manipulation was achieved by transfecting cells with the overexpression plasmids TAp63γ and TAp63γ mutant that lack p63 transactivation activity (kind gift from Dr. Kurt Engeland, University of Leipzig), or 100 nM of siRNA designed to knockdown mouse p63 expression (M-040654-01, Dharmacon, Waltham, MA, USA). As a control, cells were transfected with the corresponding empty vector plasmid (pcDNA3.1), or control siRNA containing a scrambled sequence that does not lead to specific degradation of any known cellular mRNA. Transfection efficiencies were assessed by immunoblotting analyses and found to be ~70% for JMJD3 and 80% for TAp63γ overexpression plasmids. p63 protein levels, in turn, decreased by ~ 60% after transfection with siRNAs. In experiments using the protein synthesis inhibitor CHX, cells were previously transfected with TAp63γ and either Flag-JMJD3 or Flag-JMJD3 mutant plasmids 24 h prior to addition of 50 μg⁄mL CHX (Sigma-Aldrich Co.), and maintained for 4, 6 and 8 h.

3.3.4. Total, cytosolic and nuclear protein extraction

For total protein extracts, NSCs were lysed in ice-cold buffer (10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1.5 mM potassium acetate, 1% Nonidet P-40, 2 mM dithiothreitol) and protease inhibitor cocktail tablets Complete (Roche Applied Science, Mannheim, Germany) for 30 min, and then sonicated. The lysate was centrifuged at 3,200 g for 10 minutes at 4ºC, and the supernatant recovered. For nuclear and cytosolic extracts, cells were lysed with hypotonic buffer (10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1.5 mM potassium acetate, 2 mM dithiothreitol) and protease inhibitors, homogenized with 40 strokes in a loose fitting Dounce, and centrifuged at 500 g for 10 min at 4ºC. Cytosolic proteins were recovered in the supernatant, while the nuclear pellet was washed in buffer containing 10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 0.25 M sucrose, 0.5% Triton X-100 and protease

inhibitors, then resuspended and sonicated in buffer containing 10 mM Tris-HCl, pH 7.6, 0.25 M sucrose with protease inhibitors. Finally, the suspension was centrifuged through 0.88 M sucrose at 2,000 g for 20 min at 4ºC, and nuclear proteins were recovered in the supernatant.

3.3.5. Immunoblotting

Protein levels of p63, JMJD3, Flag, β-III tubulin, NeuN, MAP2 and H3K27me3 were determined by Western blot analysis in 8 or 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis, using either primary mouse monoclonal antibodies reactive to p63 (4A4; Santa Cruz Biotechnology, Santa Cruz, CA), NeuN (MAB377; Chemicon International, Temecula, CA), Flag (M2; Sigma-Aldrich Co.), and β-III tubulin (Tuj1; Covance, Princeton, New Jersey), or primary polyclonal antibodies reactive to MAP2 (AB5622, Chemicon International) H3K27me3 (Abcam plc, Cambridge, UK) and JMJD3 (RB10082; Abgent Inc, San Diego, CA), as well as corresponding secondary antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were processed for protein detection using Super Signal^{TM} substrate (Pierce, Rockford, IL, USA). β-Actin (AC-15; Sigma-Aldrich Co.) was used as loading control. GAPDH (6C5; Santa Cruz Biotechnology) and total histone H3 (Millipore Corporation, Temecula, CA, USA) were used as markers for cytoplasmic and nuclear protein extraction, respectively. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories), according to the manufacturer's specification.

3.3.6. Immunocytochemistry

NSCs undergoing differentiation were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. Cells were then blocked for 1 h at room temperature in PBS containing 0.1% Triton-X-100, 1% fetal bovine serum and 10% normal donkey serum (Jackson Immuno Research Laboratories, Inc., West Grove, PA). Subsequently, cells were incubated with either primary monoclonal antibody reactive to p63 (4A4; 1:50) or primary polyclonal antibody reactive to JMJD3 (KDM6B; 1:50) in blocking solution, overnight at 4ºC. After three washes with PBS, cells were incubated with anti-rabbit Alexa Fluor 594- or anti-mouse Alexa Fluor 568-conjugated secondary antibodies (Invitrogen Corp.; 1:200) for 2 h at room

temperature. In control samples, the primary antibody was replaced by blocking buffer. Cells were incubated with Hoechst dye for nuclear staining. Images were acquired with an Axioskop fluorescence microscope (Carl Zeiss GmbH, Hamburg, Germany).

3.3.7. Immnunoprecipitation assay

The physical association between p63 and JMJD3 was evaluated by immunoprecipitation analysis. In brief, whole-cell extracts were prepared by lysing cells by means of sonication in lysis buffer (50 mM Tris-HCl pH 7.4, 180 mM NaCl, 1 mM EDTA, 0,5% Triton X-100) and protease inhibitors (Roche Applied Science). Immunoprecipitation experiments were carried out using the monoclonal antibody reactive to p63 (4A4) and the Ezview Red Protein G Affinity Gel (Sigma-Aldrich Co.). Typically, 500 μg of lysate was incubated with 1 μg of p63-specific antibody overnight at 4ºC. Immunoblots were then probed with the rabbit polyclonal antibody reactive to JMJD3 (KDM6B). p63 protein levels were determined in the same membrane after stripping off the immune complex for the detection of JMJD3. Immunoprecipitation assays using mouse monoclonal antibodies reactive to IgG showed neglectable binding with either JMJD3 or p63. Methylated levels of p63 were detected by immunoprecipitation analysis in denaturing conditions. In brief, whole-cell extracts were prepared by lysing cells by means of sonication in lysis buffer (50 mM Tris-HCl pH 7.4, 180 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM dithiothreitol) and protease inhibitors (Roche Applied Science). Immunoprecipitation experiments were carried out using the monoclonal antibody reactive to p63 and the Ezview Red Protein G Affinity Gel (Sigma-Aldrich Co.). Typically, 500 μg of lysate was incubated with 1 μg of p63-specific antibody overnight at 4ºC. Immunoblots were then probed with the rabbit polyclonal methylated lysine (MeK) (Abcam plc) antibody. p63 expression was determined in the same membrane after stripping off the immune complex for the detection of MeK. Finally, the results of MeK after p63 immunoprecipitation were normalized with those obtained using mouse monoclonal antibodies reactive to IgG immunoprecipitation assays as well as with p63 total levels.

3.3.8. Evaluation of apoptosis

Hoechst labeling of NSCs were used to detect apoptotic nuclei. In brief, for morphologic evaluation of apoptosis, medium was gently removed to minimize detachment of cells. Attached cells were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 10 minutes at room temperature, incubated with Hoechst dye 33258 (Sigma-Aldrich Co.) at 5 μg/ml in PBS for 5 minutes, washed with PBS and mounted using Fluoromount. Fluorescent nuclei were scored blindly and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed noncondensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation of condensed chromatin.

3.3.9. Densitometry and statistical analysis

The relative intensities of protein bands were analyzed using the QuantityOne Version 4.6 densitometric analysis program (Bio-Rad Laboratories) and normalized to the respective loading controls. Statistical analysis was performed using GraphPad InStat version 3.00 (Graph-Pad Software, San Diego, CA, USA) for the analysis of variance and Bonferroni's multiple comparison tests. Values of $p < 0.05$ were considered significant.

3.4. Results

3.4.1. TAp63γ and JMJD3 levels increase throughout mouse NSC differentiation

Several lines of evidence point toward the involvement of classical apoptotic molecules in neural differentiation, including p53, caspases and Bcl-2 family members. To investigate p63 involvement in neurogenesis, we first evaluated endogenous p63 levels throughout neural differentiation of NSCs. Comparing with the exogenous overexpression of several p63 isoforms (Fonseca et al., 2012), TAp63γ (57 kDa) was the only endogenous isoform detected by Western blot analysis in NSCs. Importantly, TAp63γ levels increased by 7-fold at 24 h of NSC differentiation (Fig. 3.1A). JMJD3 upregulation was observed as early as at 6 h of differentiation, peaking at 24 h (Fig. 3.1A), in agreement with previous studies (Fernando et al., 2005). Accordingly, JMJD3 activity was also increased during differentiation, as

demonstrated by the reduced levels of H3K27 trimethylation throughout time (Fig. 3.1A). The neuronal marker β-III tubulin increased throughout differentiation, peaking at 24 h (Fig. 3.1A), as previously described (Santos et al., 2012b).

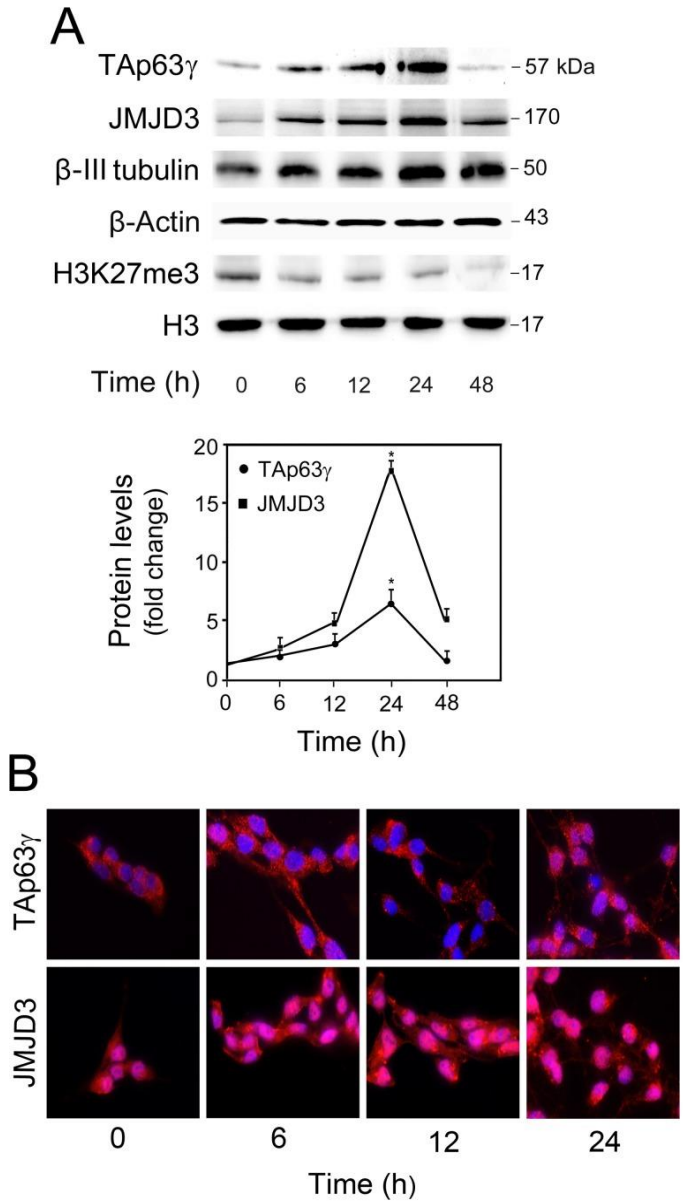


Figure 3.1. Endogenous TAp63γ protein levels increase at early stages of neural differentiation. *Undifferentiated (0 h) and differentiated mouse NSCs were collected for Western blot analysis or fixed for immunostaining at indicated time-points, as described in Materials and Methods. (A) Protein levels of TAp63γ, JMJD3, β-III tubulin, and H3K27me3 throughout NSC differentiation. Representative immunoblots (top), and TAp63γ and JMJD3 protein levels (bottom). β-Actin and H3 were used as loading controls. Immunoblots are representative of at least three different experiments. TAp63γ and JMJD3 protein levels were normalized to endogenous β-Actin protein levels and expressed as mean ± SEM for at least three different experiments. *p < 0.01 from control, undifferentiated. (B) Fluorescence staining of TAp63γ and JMJD3 throughout NSC differentiation. Hoechst dye was used for*

nuclear staining. Images are representative of at least 3 different experiments. Scale bar = 10 μm

Other markers, including mouse achaete scute homolog-1 (Mash1) and neuronal differentiation 1 (NeuroD1) were also markedly increased (data not shown). Immunocytochemistry analysis corroborated Western blot data by showing upregulation and nuclear distribution of TAp63γ and JMJD3 at 24 h of differentiation (Fig. 3.1B). These results suggest that JMJD3 and TAp63γ are coordinately regulated to influence neural-specific gene expression programs.

3.4.2. JMJD3 regulates TAp63γ protein in a demethylase activity-dependent manner

To clarify whether JMJD3 regulates TAp63γ during neurogenesis, we overexpressed JMJD3 by transfecting NSCs with a Flag-JMJD3 plasmid and evaluated TAp63γ protein levels 24 h after transfection. In control experiments, cells were transfected with either a Flag-JMJD3 mutant plasmid that does not contain the C-terminal region associated with JMJD3 demethylase activity, or no plasmid (mock). Overexpression of JMJD3 markedly increased TAp63γ protein levels when compared with control (mock) cells ($p < 0.01$) (Fig. 3.2).

To better distinguish endogenous JMJD3 protein from the transfected versions, we have also detected Flag using a Flag antibody. Of note, overexpressing the mutant form of JMJD3 had no effect on TAp63γ protein levels, indicating that JMJD3-induced increase of TAp63γ is dependent on its demethylase activity. Consistent with a role for JMJD3 as H3K27 demethylase, JMJD3 overexpression resulted in ~ 50% decreased levels of trimethylated H3K27 when compared with mock or cells transfected with JMJD3 mutant plasmid ($p < 0.05$) (Fig. 3.2). As expected, JMJD3 overexpression significantly enhanced the neuronal marker β-III tubulin by ~2-fold when compared with mock- and JMJD3 mutant plasmid-transfected cells ($p < 0.05$) (Fig. 3.2). These results are consistent with a role for JMJD3-dependent demethylation in neurogenesis progression. Moreover, the data here presented suggest that JMJD3-demethylase activity modulates TAp63γ protein levels and this may account, at least in part, for the neuronal commitment of NSCs.

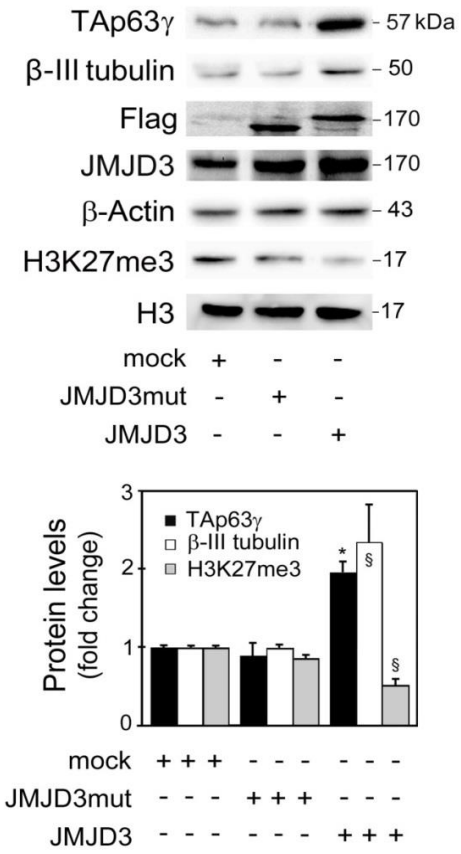


Figure 3.2. JMJD3 modulates TAp63γ levels during neural differentiation. *Mouse NSCs undergoing differentiation were transfected with JMJD3 or JMJD3 mutant (JMJD3mut), overexpression plasmids or no plasmid (mock), and collected for Western blot analysis as described in Material and Methods. Representative immunoblots of TAp63γ, β-III tubulin, Flag, JMJD3 and H3K27me3, in control (mock), JMJD3mut- and JMJD3-overexpressing cells (top) and respective protein levels (bottom). β-Actin and H3 were used as loading controls. Immunoblots are representative of at least three different experiments. TAp63γ and β-III tubulin protein levels were normalized to endogenous β-Actin whereas H3K27me3 protein levels were normalized to endogenous total H3, when compared with mock cells. Results were expressed as mean ± SEM for at least three different experiments. §p < 0.05 and *p < 0.01 from control.*

3.4.3. TAp63γ manipulation influences neuronal differentiation

To substantiate a role for TAp63γ in non-embryonic neuronal differentiation, we tested the functional relevance of TAp63γ in regulating β-III tubulin abundance in NSCs undergoing differentiation by short-interference RNA (siRNA)-mediated gene knockdown of p63. Immunoblot analysis revealed that knockdown of TAp63γ by specific p63 siRNA resulted in a ~ 50% reduction in β-III tubulin levels when compared with control siRNA-transfected cells (Fig. 3.3A and B). Other neural

markers, including the neuronal nuclei (NeuN) and microtubule-associated protein 2 (MAP2) were also markedly reduced (Fig. 3.3A).

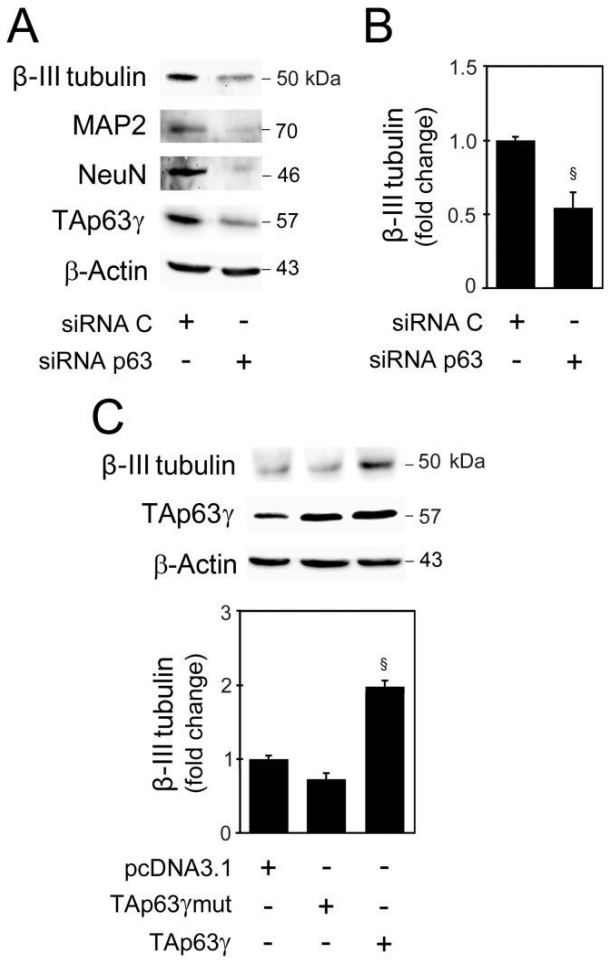


Figure 3.3. Modulation of TAp63γ levels in mouse NSCs affects neuronal differentiation. *Mouse NSCs undergoing differentiation were transfected with p63 siRNA or TAp63γ overexpression plasmids and collected for Western blot analysis as described in Material and Methods. (A) Representative immunoblot of β-III tubulin, NeuN, MAP2 and TAp63γ in cells transfected with 100 nM of either p63 siRNA or control siRNA (siRNA C) for 24 h. (B) Respective β-III tubulin protein levels. (C) Protein levels of β-III tubulin and TAp63γ in cells transfected with overexpression plasmids TAp63γ, TAp63γ mutant (TAp63γmut) or its corresponding empty vector (pcDNA3.1). Representative immunoblot of β-III tubulin and TAp63γ (top) and respective β-III tubulin protein levels (bottom). Results were normalized to endogenous β-Actin protein levels and expressed as mean ± SEM for at least three different experiments. §p < 0.05 from control.*

These results were corroborated by immunofluorescence analysis (data not shown) and indicate that the increase in TAp63γ throughout NSC differentiation is important for neuronal fate specification. In addition, overexpression of TAp63γ significantly increased β-III tubulin protein levels by ~2-fold when compared with

cells transfected with the corresponding empty vector ($p < 0.01$) (Fig. 3.3C). Importantly, overexpression with the mutant form of TAp63γ that lacks p63 transactivation activity had no effect on β-III tubulin protein levels (Fig. 3.3C), indicating that TAp63γ-mediated increase of β-III tubulin is dependent on its transactivation activity.

3.4.4. TAp63γ is directly demethylated by JMJD3 during neural differentiation

Since JMJD3 also demethylates non-histone proteins, including p53 (Solá et al., 2011b), we hypothesized that JMJD3 may possibly demethylate TAp63γ during neurogenesis. Thus, the interaction between JMJD3 and TAp63γ, as well as JMJD3-mediated TAp63γ demethylation were investigated by immunoprecipitation assays. Specifically, to evaluate TAp63γ and JMJD3 endogenous association during differentiation of NSCs, we immunoprecipitated TAp63γ from total protein extracts with p63 antibody, and immunoblots were probed with JMJD3 antibody. As depicted in Fig. 3.4A, JMJD3 directly binds to TAp63γ during NSC differentiation ($p < 0.01$).

To investigate whether TAp63γ/JMJD3 association is dependent on JMJD3-demethylase activity, TAp63γ immunoprecipitation was performed in NSCs overexpressing either JMJD3 wild-type or JMJD3 mutant plasmids. Western blot analysis revealed a significant increase in TAp63γ/JMJD3 association in cells overexpressing JMJD3 wild-type when compared with mock ($p < 0.05$) (Fig. 3.4B). Importantly, the association between TAp63γ and JMJD3 was not increased in cells transfected with mutant JMJD3, suggesting that the interaction between TAp63γ and JMJD3 is dependent on the catalytic JmjC domain of JMJD3. To investigate whether TAp63γ/JMJD3 association results in modification of TAp63γ methylation status, TAp63γ immunoprecipitation under denaturating conditions was performed in NSCs transfected with JMJD3 wild-type or JMJD3 mutant overexpression plasmids. Subsequent detection of methylated TAp63γ was performed by probing immunoblots with a pan-methylated lysine antibody. Notably, TAp63γ was significantly less methylated in cells overexpressing JMJD3 wild-type, as compared to cells transfected with the C-terminal mutant form of JMJD3 or control (mock) cells ($p < 0.01$) (Fig. 3.4C). These results suggest that the proneuronal effects of TAp63γ during differentiation of NSCs might involve direct JMJD3-dependent demethylation.

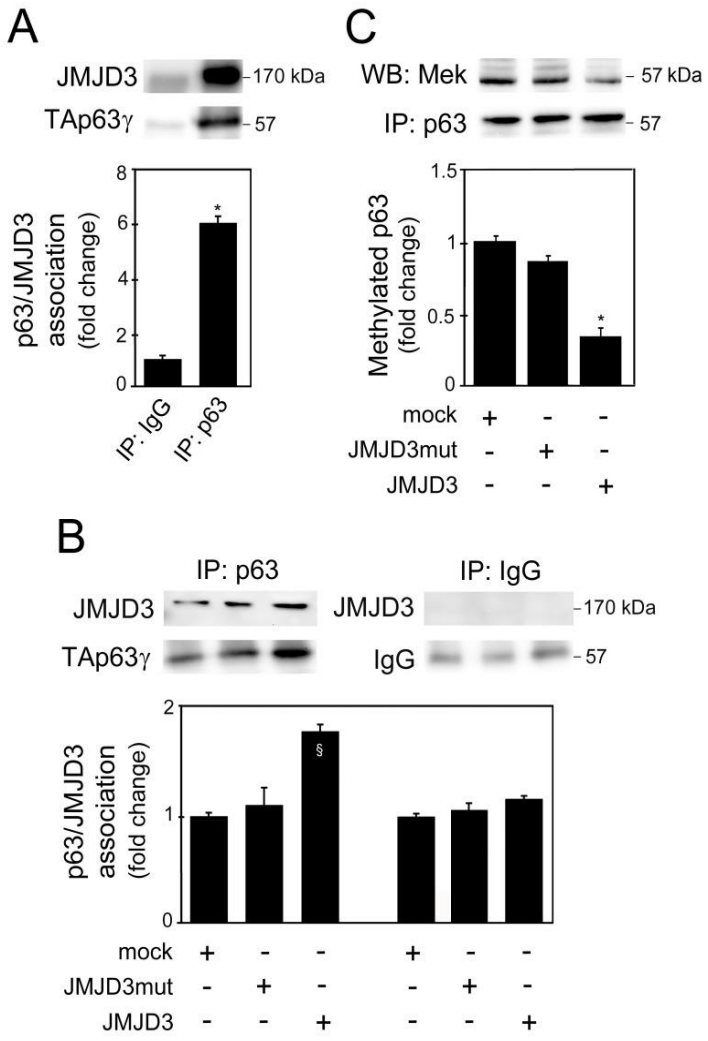


Figure 3.4. TAp63γ and JMJD3 directly associate during neural differentiation. *Mouse NSCs undergoing differentiation were collected and processed for immunoprecipitation assays with either p63 antibody or mouse IgG as control, as described in Material and Methods. (A) Representative immunoblot of JMJD3 and TAp63γ (top) and histogram of TAp63γ/JMJD3 endogenous association (bottom). (B) Representative immunoblot of JMJD3 and TAp63γ (top) and histogram of TAp63γ/JMJD3 association (bottom) in cells transfected with JMJD3 or JMJD3 mutant (JMJD3mut) overexpression plasmids, or no plasmid (mock). (C) Representative immunoblot of methylated lysine (MeK) and TAp63γ (top), and histogram of TAp63γ methylated levels (bottom). All densitometry values for JMJD3 and methylated lysines were normalized to the respective p63 expression, and the results expressed as mean ± SEM for at least three different experiments. §p < 0.05 and *p < 0.01 from controls. IP, Immunoprecipitation; WB, Western blot; MeK, Methyl K pan.*

3.4.5. TAp63γ is stabilized by JMJD3-demethylase activity and accumulates in the nucleus

p63 activity is controlled by an intricate network of PTM that targets and modulates its transcriptional activity, stability or subcellular trafficking (Stehmeier

and Muller, 2009). These PTM are catalyzed by a large number of enzymes that contribute to the activation of p63 through a variety of mechanisms. To investigate the effect of JMJD3-mediated TAp63γ demethylation on TAp63γ protein stability, we evaluated TAp63γ half-life in the presence and absence of JMJD3-demethylase activity. For this purpose, NSCs co-transfected with TAp63γ and either Flag-JMJD3, Flag-JMJD3 mutant or no plasmid (mock) were treated with the protein synthesis inhibitor CHX (Fig. 3.5).

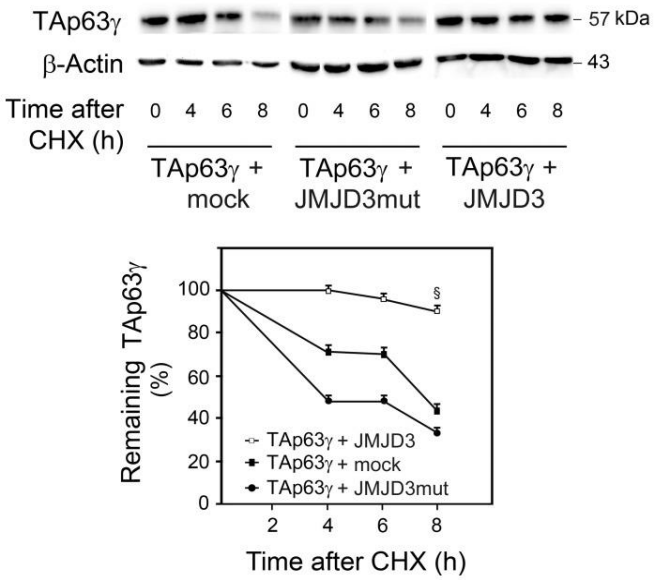


Figure 3.5. TAp63γ is stabilized by JMJD3 during mouse NSC differentiation. *Cells co-transfected with TAp63γ and with the overexpression plasmids JMJD3 (TAp63 + JMJD3), JMJD3 mutant (TAp63 + JMJD3mut), or no plasmid (TAp63 + mock) were untreated (0 h) or treated with 50 μg/mL CHX for 2, 4, 6, and 8 h. At the indicated time-points, total proteins were extracted for immunoblot analysis as described in Materials and Methods. Representative immunoblots of TAp63γ after CHX treatment (top) and percentage of remaining TAp63γ after CHX treatment (bottom). Results were normalized to endogenous β-Actin protein levels and expressed as mean ± SEM for at least three different experiments. §p < 0.05 from control.*

Our results demonstrate that TAp63γ half-life was reduced to less than 8 and 4 h, respectively in control (mock)- and mutant JMJD3-transfected cells ($p < 0.05$). In contrast, TAp63γ protein levels only slightly decreased in the presence of JMJD3 wild-type, showing that JMJD3-demethylase activity indeed regulates the stability of TAp63γ.

We next investigated the effect of JMJD3-mediated TAp63γ demethylation on TAp63γ subcellular distribution (Fig. 3.6).

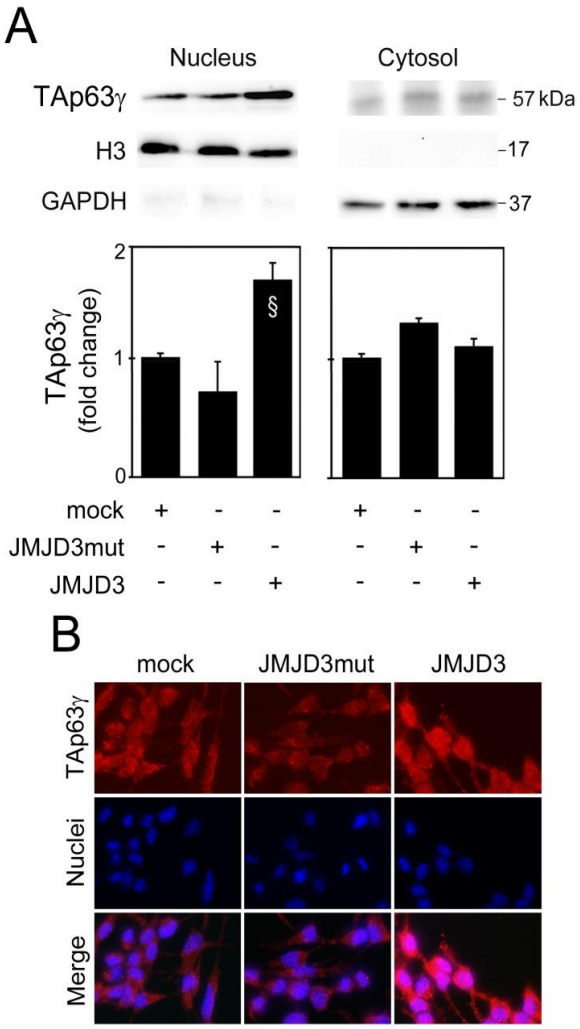


Figure 3.6. TAp63γ is translocated to the nucleus in a demethylase activity-dependent manner. *Mouse NSCs undergoing differentiation were transfected with JMJD3 or JMJD3 mutant (JMJD3mut) overexpression plasmids, or no plasmid (mock), and collected for Western blot analysis, immunocytochemistry and apoptosis assays as described in Material and Methods. (A) Representative immunoblots of nuclear and cytosolic TAp63γ (top) and respective protein levels (bottom). GAPDH and H3 were used as loading controls for cytosolic and nuclear fractions, respectively. Results were normalized to the respective endogenous controls and expressed as mean ± SEM for at least three different experiments. §$p < 0.05$ from control. (B) Subcellular localization of TAp63γ in transfected mouse NSCs. Hoechst dye was used for nuclear staining and morphologic evaluation of apoptosis. Images are representative of at least 3 different experiments. Scale bar = 10 µm.*

Increased nuclear localization of TAp63γ was observed in NSCs overexpressing wild-type JMJD3 compared with control (mock)- and mutant JMJD3-transfected cells ($p < 0.05$) (Fig. 3.6A). Immunocytochemistry analysis confirmed Western blot data by showing TAp63γ accumulation in the nucleus of JMJD3-

transfected cells compared to cells overexpressing either the mutant form of JMJD3 or control (mock) cells (Fig. 3.6B).

Together, these results suggest that TAp63γ demethylation by JMJD3 is crucial for its stabilization and accumulation in the nucleus. Moreover, these data support the hypothesis that JMJD3-induced TAp63γ nuclear translocation might direct NSCs to a neuronal fate specification, through the transcriptional activation of proneuronal genes. Importantly, TAp63γ nuclear accumulation was not associated with an increase in apoptotic cell death, as confirmed by Hoechst staining (Fig. 3.6B).

3.5. Discussion

This study demonstrates for the first time a role for p63 in adult neural progenitors, directing NSCs to a neuronal phenotype. Our findings are in accordance with postnatal p63 expression in the subventricular zone of the lateral ventricle (Hernández-Acosta et al., 2011), a region for continued adult neurogenesis. In contrast with the lack of p63 effect on central nervous system development (Hernández-Acosta et al., 2011; Holembowski et al., 2011), our results point toward an age-dependent p63 involvement in the regulation of neural differentiation. Furthermore, we demonstrate that, during neural differentiation, p63 interacts with JMJD3, a key regulator of neurogenesis (Burgold et al., 2008), which results in the modulation of p63 methylation status. Finally, our results demonstrate that JMJD3-demethylase activity regulates p63 half-life and nuclear accumulation during mouse NSC differentiation.

Much attention has been centered on p63 regulatory pathways in epidermal differentiation, due to the severe epithelial phenotype of p63 null mice (Yang et al., 1999). Recently, a role for p63 on cardiac differentiation of embryonic stem cells has also been reported (Paris et al., 2012). However, the precise function for p63 during neural differentiation remains largely unknown, when compared with other members of the p53 family. In fact, p53 and p73 are well-established key regulators of both embryonic and adult neurogenesis. Mice lacking p53 display elevated proliferation rate in the neurogenic niche of the adult lateral ventricle wall, as well as increased self-renewal of *in vitro* propagated p53 –/– NSCs (Meletis et al., 2006). Moreover, a relevant role for p53 in the biology of NSCs derived from the embryo olfactory bulb has been identified, where lack of p53 increases neurosphere-forming potential of precursor cells by favoring self-renewal capacity (Armesilla-Diaz et al., 2009).

TAp73, in turn, is an essential regulator of stemness in NSCs, which maintains an adequate neurogenic pool by promoting self-renewal and proliferation and inhibiting premature senescence of NSC and early progenitor cells, in both embryonic and adult neurogenesis (Talos et al., 2010). In addition, the width of neurogenic areas appears to be significantly reduced in brains of embryonic and adult p73 knockout mice (Agostini et al., 2010). Curiously, TAp73 acts via the basic helix-loop-helix Hey2 to promote long-term maintenance of neural precursors (Fujitani et al., 2010). p73 deficiency results in impaired self-renewal and premature neuronal differentiation of mouse neural progenitors independently of p53 (Gonzalez-Cano et al., 2010).

In contrast with p53 and p73, there is still a breach in the literature regarding p63 potential involvement in adult neurogenesis by cell death-independent mechanisms. Further, emerging knowledge suggests that a number of apoptosis-associated factors, including p53, regulate neural differentiation (Paris et al., 2012; Yang et al., 1999). This led us to explore p63 involvement in adult mouse NSC differentiation, which are capable of tripotential differentiation in neurons, glial cells and oligodendrocytes (Glaser et al., 2007), resembling adult neurogenesis. Several studies have claimed that p63 is dispensable for embryonic neurogenesis. However, since p63 knockout mice do not survive into postnatal life, a role for p63 in the adult brain cannot be excluded.

In this study, a detailed characterization of p63 isoform expression (Fonseca et al., 2012) revealed that only one isoform with ~57 kDa was detected and subsequently confirmed as TAp63γ by comparison with exogenous p63 isoform overexpression (Jacobs et al., 2005). Time-course analysis of TAp63γ protein levels throughout NSC differentiation demonstrated an increase of TAp63γ at early stages of neural differentiation, when JMJD3 protein levels and activity were already significantly increased. Accordingly, we have previously demonstrated that JMJD3 expression and activity at the Hoxc8 promoter increased early in mouse NSC differentiation (Solá et al., 2011b). Importantly, overexpression of JMJD3 in NSCs undergoing differentiation resulted in increased levels of TAp63γ protein, accompanied by increased neuronal marker β-III tubulin. We have previously reported a similar effect of JMJD3 on the neural precursor Pax6 in JMJD3-transfected mouse NSCs (Solá et al., 2011b). All together, these results suggest that JMJD3 modulates TAp63γ levels during neural differentiation, which might contribute to the neuronal fate specification of NSCs. In accordance with this hypothesis, overexpression of TAp63γ during early

differentiation of NSCs resulted in increased β-III tubulin levels, whereas downregulation of TAp63γ expression by specific p63 siRNA abrogated the increase in β-III tubulin.

Interestingly, in NSCs undergoing differentiation, both TAp63γ and β-III tubulin were modulated by JMJD3 in a demethylase activity-dependent manner. While not excluding an effect of H3K27 demethylation on NSC neuronal differentiation, our results also suggest that TAp63γ-mediated neuronal differentiation essentially relies on TAp63γ direct demethylation by JMJD3. In this respect, JMJD3 was reported to bind non-histone proteins, including p53 (Solá et al., 2011b), much like other demethylases (Tsai et al., 2008). Our results demonstrate a direct interaction between TAp63γ and JMJD3, dependent of the C-terminal region of JMJD3, and modulation of TAp63γ methylation levels by JMJD3. Importantly, JMJD3-demethylase activity appears to be relevant in regulating TAp63γ protein half-life. Cycloheximide experiments demonstrated that modulation of TAp63γ levels by JMJD3 during differentiation of NSCs occurs through protein stabilization and not by transcriptional regulatory mechanisms. Supporting this idea, it has recently been shown that among several genes involved in neurogenesis and neuronal differentiation that are up-regulated by H3K27 demethylation, p63 is not included (Pereira et al.). This suggests that JMJD3-mediated regulation of TAp63γ in NSCs does not occur at the transcriptional level. In addition to TAp63γ stabilization, we here show that JMJD3-demethylase activity increases TAp63γ nuclear accumulation during NSC differentiation. These results are in agreement with those previously obtained for JMJD3 and p53 in mouse NSCs (Solá et al., 2011b), reinforcing the similarities in structural and function properties of p53 family members. Noteworthy, TAp63γ cellular distribution concurs with a role for this protein as a transcriptional activator of pro-neuronal gene expression. Accordingly, NSCs cells transfected with the mutant form of TAp63γ that lacks p63 transactivation activity failed to differentiate in β-III tubulin-positive cells, indicating that TAp63γ-mediated neuronal differentiation might be dependent on its transactivation activity. This study constitutes the first to show that TAp63γ demethylation affects its stability and subcellular localization. Similar effects were already described for p63 phosphorylation and acetylation, the best characterized p63 PTM (Collavin et al., 2010) resulting in different cellular outcomes. A deepest knowledge of these regulatory modifications of p63 protein will improve our understanding of p63

function during adult neural differentiation. In this respect, future studies are warranted to determine the specific residues demethylated by JMJD3 and to address the importance of TAp63γ demethylation in neurogenesis by modification of the identified residues. Since most mutated p63 proteins found in patients suffering from ectodermal dysplasia syndromes (Ying et al., 2005) are more stable than wild-type peptides, it would be interesting to clarify whether the mutated residues are sites of methylation. We also anticipate TAp63γ protein demethylation to be highlighted as a PTM crucial for the neurogenic process.

In conclusion, our results clearly demonstrate that TAp63γ and JMJD3 directly interact during NSC differentiation, in a JMJD3 C-terminal region-dependent manner. In addition, both TAp63γ protein stability and nuclear accumulation appear to be modulated by JMJD3-demethylase activity during neural differentiation. These findings suggest that JMJD3 is a regulator of TAp63γ during NSC differentiation, and JMJD3-mediated TAp63γ demethylation plays a role in proneural gene expression.

Acknowledgments

We thank Dr. Kurt Engeland (University of Leipzig, Germany) for the kind gift of the TAp63γ and TAp63γ mutant overexpression plasmids, and Dr. Kristen Jepsen (University of California, San Diego, USA) for the Flag-JMJD3 and Flag-JMJD3 mutant overexpression plasmids.

Amyloid β Peptides Promote Autophagy-dependent Differentiation of Mouse Neural Stem Cells

Maria B. Fonseca1, Susana Solá[1,2], Joana M. Xavier[1], Pedro A. Dionísio[1], Cecília M. P. Rodrigues[1,2]

[1]*Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL), and*
[2]*Department of Biochemistry and Human Biology, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal*

Molecular Neurobiology 2013; in press.

Reprinted from *Molecular Neurobiology*, (#MOLN-D-13-00031R1), Maria B. Fonseca, Susana Solá, Joana M. Xavier, Pedro A. Dionísio, Cecília M. P. Rodrigues. Amyloid β peptides promote autophagy-dependent differentiation of mouse neural stem cells., Copyright 2013, with permission from Springer Science plus Business Media. All rights reserved.

4.1. Abstract

Although regarded as neurotoxic, amyloid β (Aβ) peptides may also mediate a wide range of non-pathogenic processes. Autophagy has been implicated in Aβ-mediated effects, although its precise function in neural differentiation remains unknown. Here, we addressed the role of different Aβ fragments in neural stem cell (NSC) proliferation and differentiation, and investigated whether autophagy is involved in Aβ-induced alterations of neural fate. Our results demonstrate that neuronal and glial-specific protein markers are significantly induced by both $A\beta_{1-40}$ and $A\beta_{1-42}$. However, $A\beta_{1-40}$ preferentially enhances neurogenesis of NSCs, as determined by βIII-tubulin, NeuN and MAP2 neuronal marker immunoreactivity, while $A\beta_{1-42}$ appears to favor gliogenesis. In contrast, $A\beta_{25-35}$ does not influence NSC fate. The effect of $A\beta_{1-40}$ on neurogenesis is partially dependent on its role in NSC self-renewal as both S-phase of the cell cycle and BrdU labeling were markedly increased. Nevertheless, $A\beta_{1-40}$ resulted also in increased Tuj1 promoter activity. Autophagy, assessed by conversion of endogenous LC3-I/-II, fluorescence of pGFP-LC3 transfected cells, and Atg9 protein levels was evident in both $A\beta_{1-40}$- and $A\beta_{1-42}$-treated NSCs, independently of reactive oxygen species production and apoptosis. Finally, inhibition of autophagy by pharmacologic means abrogated Aβ-induced lineage-specific protein markers. These results support distinct roles for different Aβ peptides in NSC fate decision and underline the importance of autophagy control of this process.

Keywords: Alzheimer's disease; Gliogenesis; Neural stem cell fate; Neurogenesis; Proliferation

4.2. Introduction

Over the past years, neural differentiation in mammalians has significantly impacted on our understanding of brain physiology, holding great promise for neuro-replacement therapies (Alvarez-Buylla and Garcia-Verdugo, 2002; Bernal and Peterson, 2004; Gage, 2000; Kempermann and Gage, 2000). Neural stem cells (NSCs) are self-renewing, multipotent cells that have the ability to differentiate into the three major cell types of the central nervous system (CNS) including, neurons, astrocytes and oligodendrocytes (Reynolds and Weiss, 1996). The dimension of the NSC pool and its ability to restore damaged neural tissue in the brain results from tightly regulated cellular processes, including self-renewal, differentiation and cell death. Thus, a better understanding of the molecular mechanisms responsible for neural differentiation, as an alternative to cell death will surely advance neuronal repair strategies.

Amyloid-β (Aβ) peptide is the main component of deposits found in the brains of patients with Alzheimer's disease (AD) (Selkoe, 2001b). It is processed from the amyloid precursor protein (APP), through β- and γ-secretases, originating $A\beta_{1-40}$, the major species under physiological conditions, and a number of minor and more toxic species, such as $A\beta_{1-42}$ (Steiner and Haass, 2000). While best known as components responsible for neuronal death in AD, evidence has suggested that Aβ species are highly multifunctional peptides with significant non-pathological activities. In fact, under deprived conditions, Aβ exhibits neurotrophic and neuroprotective properties at physiological concentrations in neonatal cells (Kontush et al., 2001; Whitson et al., 1989; Yankner et al., 1990). $A\beta_{1-40}$ might have a beneficial role in reducing the amyloid burden, while $A\beta_{1-42}$ is the major component of amyloid deposits, responsible for many neurodegenerative phenotypes and learning deficits (McGowan et al., 2005). Indeed, studies in human brain, cerebrospinal fluid and plasma, as well as in transgenic animals and cellular systems modeling familial AD mutations have demonstrated that the $A\beta_{42}/A\beta_{40}$ ratio is consistently elevated (Bentahir et al., 2006; Kumar-Singh et al., 2006). Recently, it has been demonstrated that $A\beta_{1-40}$ may also have neurogenic properties (Chen and Dong, 2009). However, the precise molecular pathways underlying the regulation of NSC proliferation and differentiation by different Aβ peptides species are still not fully defined, and may implicate survival among other processes.

Autophagy as a dynamic process of protein degradation fulfills multiple functions in mammalian cells, and is essential for cellular survival and differentiation,

development and homeostasis (Asanuma et al., 2003; Levine and Klionsky, 2004; Pua et al., 2007; Zeng and Zhou, 2008; Zhao et al., 2010; Zhuang et al., 2011). It is regulated by a complex signal transduction machinery involving autophagy-related (Atg) gene products and other proteins that sense the intracellular energy status. As most neurons survive for the lifetime of the organism, maintenance of organelle function and clearance of aberrant or damaged proteins are critical processes regulated by autophagy. In fact, a growing number of studies support the idea that autophagy is highly active during differentiation and development (Asanuma et al., 2003; Vessoni et al., 2012), including neural differentiation (Zhao et al., 2010). In addition, autophagy has recently been reported as a mechanism of neuronal protection from Aβ-induced cytotoxicity (Cheung et al., 2011; Hung et al., 2009; Moreira et al., 2010; Wang et al., 2010). Although Aβ-induced cell death via an autophagic degradation pathway has also been reported, the correlation between pro-neurogenic effects of Aβ peptides and autophagic activity has not been explored before.

Reactive oxygen species (ROS) may be generated by Aβ species. Further, ROS are important signaling molecules for autophagy (He et al., 2011), during periods of nutrient deprivation, hypoxia, ischemia, reperfusion injury, and general cell stress (Chen et al., 2009; Gurusamy and Das, 2009), and have been implicated in differentiation in certain developmental programs such as neurogenesis (Essick and Sam, 2010). In fact, the correct balance of ROS production and utilization is critical to the regulation of self-renewal and differentiation in pluripotent cells (Ray et al., 2012; Santos et al., 2012a).

In the present study, we elucidated the role of different Aβ peptides during NSC fate decision. Our results demonstrate that, in contrast with their conventional effects in cell death, lower levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ mediate neural differentiation. $A\beta_{1-40}$ strongly promotes NSC proliferation and neurogenesis, while $A\beta_{1-42}$ facilitates glial fate decision. Moreover, Aβ active fragments increase neural-lineage markers possibly by enhancing the autophagy survival response in differentiating NSC, independently of ROS production and apoptosis.

4.3. Material and methods

4.3.1. Cell culture

A mouse NSC line was derived from day 14.5 post-coitum mouse fetal forebrain. This cell line was established using a method that produces pure cultures of adherent NSCs, which continuously expand by symmetrical division and are capable of

tripotential differentiation (Ezashi et al., 2005; Smith et al., 2000). NSCs were grown in monolayer as previously described (Conti et al., 2005; Glaser et al., 2007; Pollard et al., 2006), and routinely maintained in undifferentiation medium, Euromed-N medium (EuroClone S.p.A., Pavia, Italy), supplemented with 1% N-2 supplement (Invitrogen Corp., Grand Island, NY, USA), 20 ng/mL epidermal growth factor (EGF; PeproTech EC, London, UK), 20 ng/mL basic fibroblast growth factor (bFGF; PeproTech EC) and 1% penicillin-streptomycin (Invitrogen Corp.), in uncoated tissue culture plastic flasks at 37°C, in a humidified atmosphere of 5% CO_2. Medium was changed every 3 days and cells collected with accutase (Sigma-Aldrich Co., St. Louis, MO) when confluent. Differentiation of NSCs was performed by first platting cells in undifferentiation medium onto uncoated tissue culture plastic dishes at $1x10^6$ cells/cm^2 for 24 h, and changing the culture medium to differentiation medium, Euromed-N medium supplemented with 10 ng/mL bFGF, 0.5% N-2 supplement, 1% B27 supplement and 1% penicillin-streptomycin.

4.3.2. Cell treatments

Twenty-four hours after induction of differentiation, 1.5 μM of peptide active fragments $A\beta_{25-35}$, $A\beta_{1-40}$, or $A\beta_{1-42}$ (American Peptide Company, Sunnyvale, CA, USA) were added to the culture medium for 48 h. In selected experiments, undifferentiated cells were also incubated with Aβ peptide fragments for different time periods, up to 24 h. Aβ-treated cells were then collected for luciferase and lactate dehydrogenase (LDH) assays, immunoblotting, flow cytometry, and immunocytochemistry analysis. To inhibit autophagy, 10 mM of 3-methyladenine (3-MA) (Sigma-Aldrich Co.) was added to the culture medium for 48 h.

4.3.3. Plasmid transfections

For the luciferase reporter assay, cells were transfected 24 h after plating into 35 mm dishes with the murine Tuj1-promoter plasmid (1 μg per dish) and the *Renilla luciferase* reporter as internal control for transfection efficiency (1 ng per dish) (CMV-PRL; Promega, Fitchburg, WI, USA) using lipofectamine 2000 (Invitrogen Corp.). The Tuj1-promoter, kindly provided by Fred Gage, was cloned by PCR from genomic DNA and inserted into pNeoLuci (Spiliotopoulos et al., 2009). To analyze the intracellular localization and processing of the microtubule-associated protein 1 light chain 3 (LC3), a reliable autophagy marker, cells were transfected with a pGFP-LC3 plasmid for 24 h.

The pGFP-LC3 construct was kindly provided by Dr. Tamotsu Yoshimori and generated by inserting LC3 cDNA into the *Bgl*II and *Eco*RI sites of pEGFP-C1, a GFP fusion protein expression vector (Clontech Laboratories Inc., Mountain View, CA, USA) (Kuwabara et al., 2004). GFP intensity was evaluated by immunocytochemistry.

4.3.4. Dual luciferase assay

Twenty-four hours after transfection with the murine Tuj1-promoter plasmid, cells were treated with Aβ peptides in fresh culture medium for 48 h before harvest. Firefly and *Renilla luciferase* activities were measured using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's recommendation. The luminescence signal of the specific luciferase reporter plasmid was normalized to the internal control *Renilla luciferase* reporter intensity.

4.3.5. Immunoblotting

Steady-state levels of βIII-tubulin, GFAP, NeuN, MAP2, Atg9 and LC3 in NSCs were determined by Western blot analysis. Cells were collected and lysed for isolation of total protein extracts using ice-cold lysis buffer (10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1.5 mM potassium acetate, 1% Nonidet P-40, 2 mM dithiothreitol) and protease inhibitor cocktail tablets Complete (Roche Applied Science, Mannheim, Germany) for 30 min. Samples were then sonicated and centrifuged at 3,200*g* at 4ºC for 10 min. Protein content was measured by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's specifications. One hundred μg of protein extracts were separated on 8 and 12% sodium dodecyl sulphate polyacrylamide electrophoresis gels. After electrophoretic transfer onto nitrocellulose membranes, and blocking with a 5% milk solution, membranes were incubated overnight at 4ºC with either primary mouse monoclonal antibodies reactive to βIII-tubulin (Tuj1 clone; Covance, Princeton, New Jersey), GFAP and NeuN (MAB360 and MAB377, respectively; Chemicon International, Temecula, CA, USA), primary rabbit polyclonal antibodies reactive to MAP2 (AB5622; Chemicon International) and LC3 (PA1-16931; Affinity Bioreagents, Golden, Colorado, USA), or primary goat polyclonal antibody reactive to Atg9a (sc-70141; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Blots were subsequently incubated with secondary antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories) for 3 h at room temperature. Membranes were processed for protein detection using Super Signal™ substrate

(Pierce, Rockford, IL, USA). β-Actin (AC-15; Sigma-Aldrich Co.) was used as loading control.

4.3.6. Immunocytochemistry

For detection of proliferating cells, S-phase nuclei were stained in undifferentiated cells using the bromodeoxyuridine (BrdU) *in-situ* detection kit (BD Biosciences Pharmingen, San Diego, CA, USA). BrdU was added to the culture medium 18 h after Aβ treatments, and cells were re-incubated for additional 6 h and processed according to manufacturer's instructions. For fluorescence microscopy, NSCs cells were maintain in differentiation medium for 6 days, fixed with 4% paraformaldehyde (w/v) in phosphate buffer saline (PBS) and blocked for 1 h at room temperature in PBS, containing 0.1% Triton X-100, 1% fetal bovine serum (FBS), and 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Cells were then incubated with either anti-βIII-tubulin antibody at a dilution of 1:500 in blocking solution, with anti-MAP2 (AB5622, Millipore Corporation, Darmstadt, Germany), anti-Nestin (MAB353; Chemicon International), anti-NG2 (neuronal/glial 2 AB5320; Millipore) and anti-GFAP antibodies at a dilution of 1:200 in blocking solution, or with anti-GFP (B-2) antibody (sc-9998; Santa Cruz Biotechnology, Inc.) at a dilution of 1:50 in blocking solution, overnight at 4°C. At the end of the incubation with primary antibodies and after washing in PBS, cells were incubated with appropriate secondary antibodies, mouse antibodies conjugated to either Alexa 568 (Life Technologies Ltd., Carlsbad, CA, USA) at a dilution of 1:200 or Dylight 488 (Jackson ImmunoResearch Laboratories, Inc.) at a dilution 1: 100, and Alexa 594-conjugated anti-rabbit antibody (Life Technologies Ltd) at a dilution of 1:200, for 2 h at room temperature. Finally, nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich Co.) at 50 μg/ml in PBS, for 5 min at room temperature. Samples were mounted using Fluoromount-G™ (Beckman Coulter, Inc., Brea, CA, USA). Fluorescence microscopy assessments were performed with a Zeiss AX10 microscope (Carl Zeiss, Jena, Germany) equipped with a Leica DFC490 camera (Leica Wetzlar, Germany) or with a Zeiss LSM 510 META confocal microscope (Carl Zeiss).

4.3.7. Flow cytometry analysis

For detection of βIII-tubulin and GFAP expression levels, cells were fixed with 4% paraformaldehyde in PBS for 20 min at 4^{o}C, washed twice with washing solution

0.1% saponin (Fluka, Biochemika, Switzerland) in PBS, and blocked for 20 min in blocking solution 0.25% saponin and 5% FBS in PBS. Subsequently, cells were washed and incubated with antibodies reactive to βIII-tubulin or GFAP at a dilution of 1:500 and 1:200, respectively, in antibody blocking solution 0.1% saponin and 5% FBS in PBS, for 30 min. Cells were then washed twice and incubated with anti-mouse antibody conjugated to Dylight 488, at a dilution of 1:100 for 30 min. Cells were washed twice, resuspended in PBS with 2% FBS and analyzed using the Guava EasyCyte 5HT® Cytometry system (Millipore). For ROS quantification, 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Molecular Probes, Invitrogen, Eugene, OR, USA) was added to the culture medium 30 min prior to cell collection. Then, cells were ressuspended in PBS with 2% FBS, and emission of green fluorescence was analyzed in the Guava EasyCyte 5HT® Cytometry system after 20 min. Proliferation levels were determined by BrdU incorporation analysis using the APC BrdU Flow Kit (BD Biosciences Pharmingen). BrdU was added to the culture medium 18 h after cell treatments, and cells were re-incubated for additional 6 h and later analyzed by flow cytometry using the LSR Fortessa^{TM} system (Becton Dickinson, Mountain View, CA). The percentage of cells in the S-phase of the cell cycle was also evaluated by the LSR Fortessa^{TM} system. First, cells were synchronized by double-thymidine block. Twelve hours after plating, 2 mM thymidine (Sigma-Aldrich Co.) was added to the culture media for additional 12 h. Cells were then released from first thymidine block by removing culture media, washing 3 times with PBS and by adding fresh undifferentiation medium, without thymidine. Twelve hours later, cells were submitted to a second thymidine block. Aβs were added to the medium without thymidine at the end of second block. Cells were grown for additional 48 h, prior to cell collection with accutase. Subsequently, cells were washed with PBS, fixed in 80% ethanol at 4ºC overnight, and then stained with 5 μg/mL propidium iodide (PI) (Sigma-Aldrich Co.) for DNA contents. Data were statistically evaluated using FlowJo software (Tree Star, Inc, Ashland, OR, USA).

4.3.8. Evaluation of cell death

Hoechst labeling of NSC was used to detect apoptotic nuclei. In brief, for morphologic evaluation of apoptosis, medium was gently removed to minimize detachment of cells. Attached cells were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature, incubated with Hoechst dye 33258 (Sigma-Aldrich

Co.) at 5 μg/ml in PBS for 5 min, washed with PBS and mounted using Fluoromount. Fluorescent nuclei were scored blindly and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed noncondensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation of condensed chromatin. Cell viability was quantified by the LDH assay (Sigma-Aldrich Co.). The absorbance was measured spectrophotometrically at 490 nm wavelength, using a 96-well plate reader (Bio-Rad Laboratories).

4.3.9. Densitometry and statistical analysis

The relative intensities of protein bands were analyzed using the QuantityOne Version 4.6 densitometric analysis program (Bio-Rad Laboratories) and normalized to the respective loading controls. Statistical analysis was performed using GraphPad InStat version 3.00 (Graph-Pad Software, San Diego, CA, USA) for the analysis of variance and Bonferroni's multiple comparison tests. Values of $p < 0.05$ were considered significant.

4.4. Results

4.4.1. Characterization of NSCs isolated from mouse fetal forebrain under differentiating conditions

Monolayer culturing systems of NSCs can be generated from embryonic stem cells, induced pluripotent stem cells (derived from reprogrammed somatic cells), as well as from fetal and adult brain (Kabeya et al., 2000). These systems have the potential to give rise to neurons, astrocytes and oligodendrocytes. The different cellular compositions of the monolayer cultures, with homogeneous composition, result in high neurogenic potential. In this study, we used NSCs derived from 14.5-days post coitum mouse fetal forebrain. This cell line was established using a method that produces pure cultures of adherent NSCs, which continuously expand by symmetrical division and are capable of tripotential differentiation (Conti and Cattaneo, 2010). NSCs were grown in monolayer as previously described (Conti et al., 2005; Glaser et al., 2007; Pollard et al., 2006) and routinely maintained in undifferentiating medium (Fig. 4.1A). Under these conditions, ~ 90% of the cells were positive for proliferative marker Nestin.

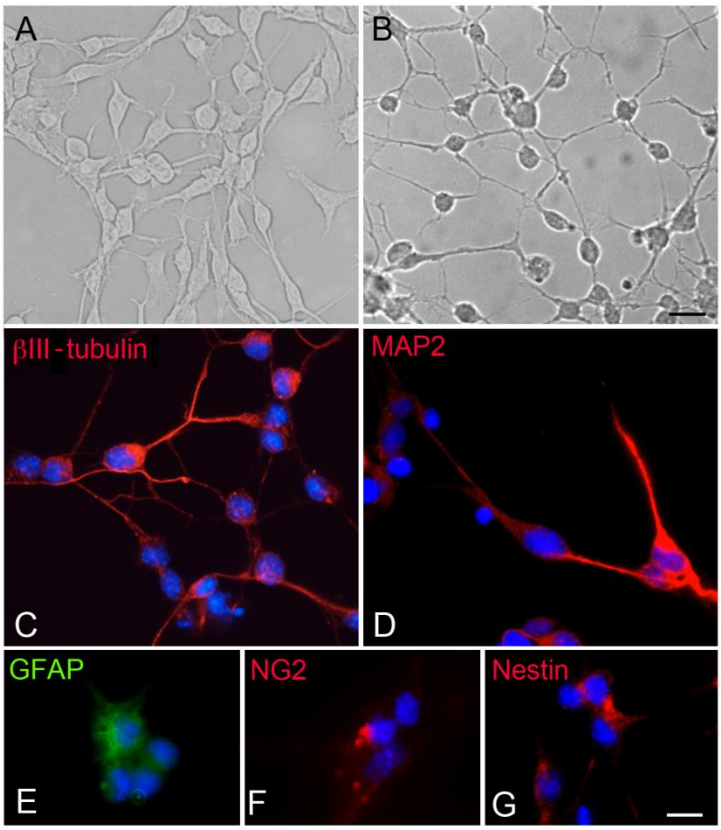


Figure 4.1. Monolayer culture system of NSCs and differentiation into major CNS cell types. *Mouse NSCs were expanded and induced to differentiate as described in Materials and Methods. Undifferentiated (A) and differentiated (B) NSCs. Fluorescence staining of βIII-tubulin (C), MAP2 (D), GFAP (E), NG2 (F) and Nestin (G) throughout NSC differentiation. Nuclei were stained with Hoechst. Scale bar, 10 µm.*

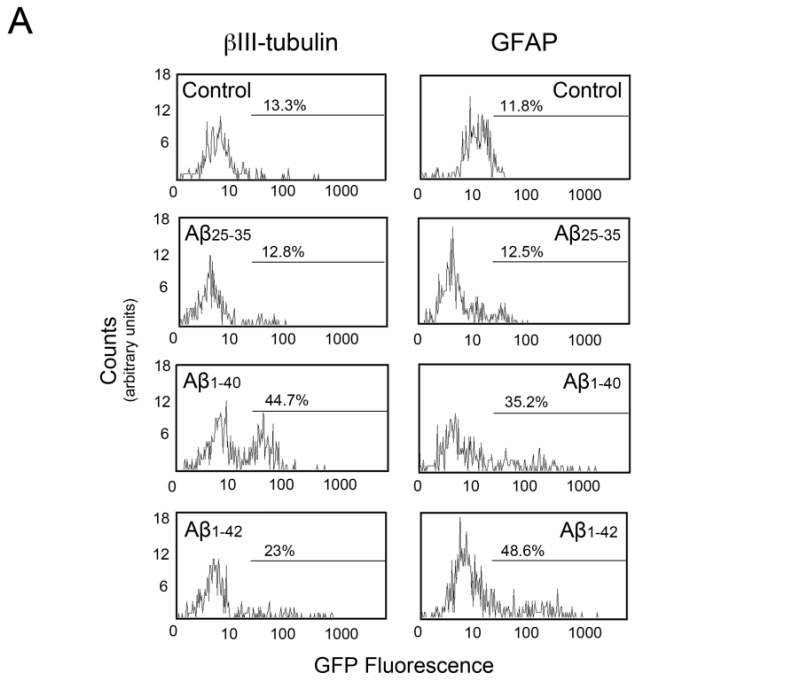
Following incubation in differentiation medium, cells displayed a sharp increase in markers for neurons and neural precursors. In fact, at 3 days of differentiation, 15-20% of cells were positive for βIII-tubulin or GFAP, and ~ 50% were positive for Nestin. After 3 days in differentiating conditions, cells were gently dissociated, replated on a laminine-coated surface, and exposed to a FGF-2 and BDNF containing medium for terminal differentiation for additional 3 days (Spiliotopoulos et al., 2009). When exposed to these conditions, NSCs gradually adhered and progressively developed a neuronal morphology as indicated by the appearance of neurites outgrowth, and establishment of complex branched morphology and cell-cell contacts (Fig. 4.1B). A significant fraction of cells in culture showed increased immunoreactivity for the immature neuronal marker βIII-tubulin, in addition to acquiring immunoreactive signal for the mature neuronal marker MAP2, and showing a more complex and branched neuronal morphology (Fig. 4.1C and D). Finally, only a small portion of the total cell population became GFAP-positive astrocytes and NG2-positive oligodendrocytes (Fig.

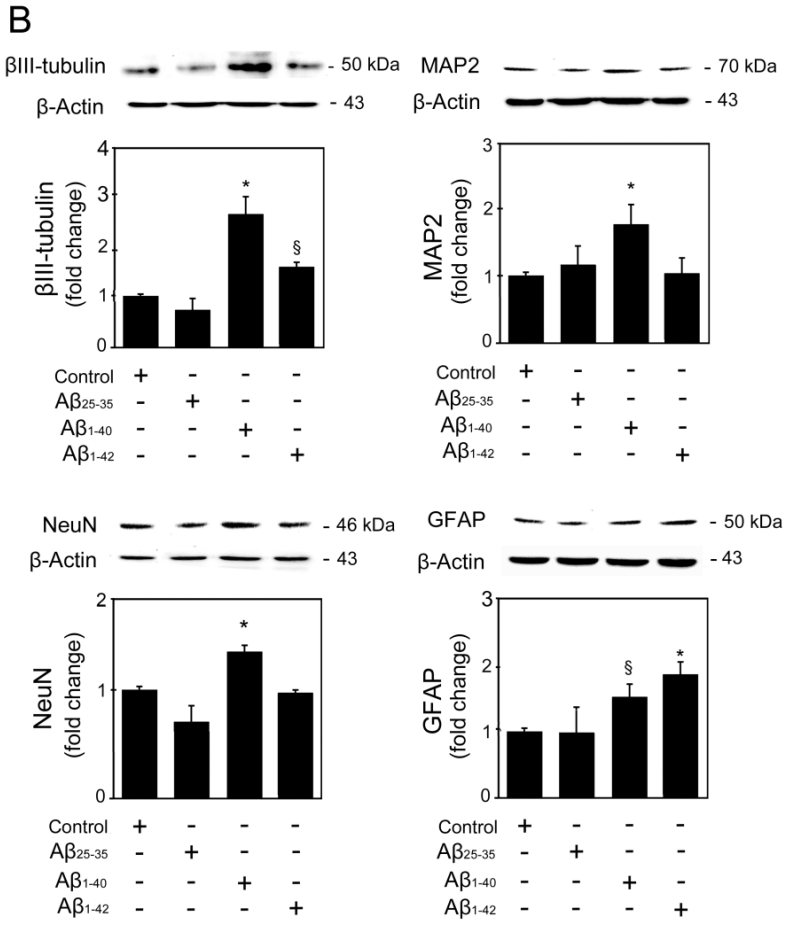
4.1E and F), indicating that the culture conditions do not favor conversion into the astroglial and oligodendroglial lineages. Differentiated cells also appear to have cell type-specific morphologies. Consistent with induction of differentiation, Nestin immunoreactivity in NSC cultures showed a few number of cells expressing this immature marker (Fig. 4.1G).

4.4.2. Distinct roles of $A\beta_{1-40}$ and $A\beta_{1-42}$ in modulating lineage-specific markers of NSCs

Several lines of evidence point toward the involvement of Aβ in neurogenesis (Spiliotopoulos et al., 2009). However, the effects of Aβ in neural differentiation have not reached a consensual agreement. To address the role of Aβ during neural differentiation *in vitro*, the expression levels of βIII-tubulin, MAP2, NeuN and GFAP were investigated in NSCs incubated with $A\beta_{1-40}$, $A\beta_{1-42}$ or $A\beta_{25-35}$ for 48 h. Flow cytometry analysis showed that $A\beta_{1-40}$ and $A\beta_{1-42}$ induced a marked increase of both βIII-tubulin- and GFAP-positive cells ($p < 0.01$). However, while $A\beta_{1-40}$ appeared to preferentially increase neuronal markers, $A\beta_{1-42}$ exhibited a stronger effect on astrocytic differentiation (Fig. 4.2A). In addition, $A\beta_{25-35}$ elicited no significant alterations in neuronal and astrocytic-specific protein markers. Accordingly, Western blot data revealed that, although increasing GFAP levels ($p < 0.05$), $A\beta_{1-40}$ markedly augmented βIII-tubulin, MAP2 and NeuN ($p < 0.01$) throughout differentiation (Fig. 4.2B).

$A\beta_{1-42}$, in turn, induced βIII-tubulin levels ($p < 0.05$); however, the strongest effect was observed in GFAP protein levels, which doubled in $A\beta_{1-42}$-treated cells ($p < 0.01$). Finally, immunocytochemistry analysis of the βIII-tubulin and MAP2 markers corroborated the effects of $A\beta_{1-40}$ and $A\beta_{1-42}$ in regulating neuronal lineage of NSCs (Fig. 4.2C). While $A\beta_{1-42}$ slightly increased the expression of βIII-tubulin and did not alter MAP2, $A\beta_{1-40}$ significantly increased both markers, when compared with untreated cells. These results reinforce a stronger effect of $A\beta_{1-40}$ in enhancing the neuronal lineage of NSCs when compared with $A\beta_{1-42}$, and demonstrate distinct effects for different Aβ active fragments in NSC-fate determination.


A
βIII-tubulin
GFAP
Control
13.3%
11.8%
Aβ25-35
12.8%
12.5%
Aβ1-40
44.7%
35.2%
Aβ1-42
23%
48.6%
Counts
(arbitrary units)
GFP Fluorescence


B
βIII-tubulin
- 50 kDa
β-Actin
- 43
MAP2
- 70 kDa
βIII-tubulin (fold change)
MAP2 (fold change)
Control
Aβ25-35
Aβ1-40
Aβ1-42
NeuN
- 46 kDa
GFAP
- 50 kDa
NeuN (fold change)
GFAP (fold change)

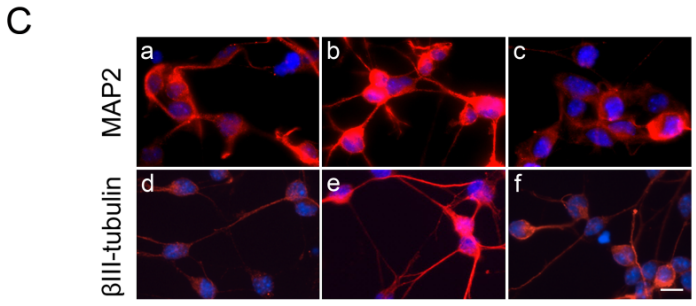

C
MAP2
βIII-tubulin
a
b
c
d
e
f

Figure 4.2. Aβ1-40 preferentially promotes neuronal differentiation of NSCs. *Cells were induced to differentiate, treated with either $A\beta_{25-35}$, $A\beta_{1-40}$ or $A\beta_{1-42}$ for 48 h, and labeled for neural marker detection by flow cytometry as described in Materials and Methods. (A) Representative histograms of βIII-tubulin-positive cells (left) and GFAP-positive cells (right). (B) Representative immunoblot of βIII-tubulin and MAP2 (top), and NeuN and GFAP (bottom), and respective protein levels. Results were normalized to endogenous β-Actin protein levels and expressed as mean ± SEM for at least three different experiments. (C) Immunostaining of neuronal markers βIII-tubulin and MAP2 in control untreated cells (a and d), and in cells treated with $A\beta_{1-40}$ (b and e) and $A\beta_{1-42}$ (c and f). Nuclei were stained with Hoescht. Scale bar, 10 µm. *p < 0.01 and §p < 0.05 from controls.*

4.4.3. $A\beta_{1-40}$ increases proliferation and neuronal-promoter activity of NSCs

It has been suggested that Aβ peptides may also alter NSC proliferation depending on the time and type of animal models used (Chen and Dong, 2009). We next investigated whether the effects of $A\beta_{1-40}$ and $A\beta_{1-42}$ in enhancing lineage-specific markers were due to proliferative effects. Undifferentiated cells were preincubated with $A\beta_{1-40}$ and $A\beta_{1-42}$ for 24 h and pulse-labeled for 6 h with BrdU to mark S-phase cells. Immunocytochemistry analysis revealed that treatment with $A\beta_{1-40}$ significantly increased BrdU incorporation, when compared with untreated cells (Fig. 4.3A). Surprisingly, no significant changes were observed in the percentage of BrdU-positive cells upon $A\beta_{1-42}$ treatment. The percentage of BrdU incorporated in Aβ-treated NSCs was also determined by flow cytometry analysis. Our results confirmed that ~ 80% of the untreated cells incorporated BrdU, indicating that the majority of NSCs are in S-phase and proliferating. Interestingly, > 90% of the cells treated with $A\beta_{1-40}$ were BrdU-positive, while treatment with $A\beta_{1-42}$ had no effect in BrdU incorporation, when compared with untreated cells ($p < 0.05$) (Fig.4.3B). To validate our results, the DNA content distribution of NSCs was also analyzed by flow cytometry.

In these experiments cells were stained with PI for determining DNA content after treatment with Aβ peptides. As shown in Fig.4.3C, the percentage of cells in S-phase raised ~ 15% after $A\beta_{1-40}$ treatment ($p < 0.05$). As expected, no significant increase in proliferation was observed following treatment with $A\beta_{1-42}$. Thus, our results suggest that $A\beta_{1-40}$ and $A\beta_{1-42}$ have distinct functions in mediating NSC proliferation. While the effect of $A\beta_{1-40}$ on neuronal-specific lineage may be partially the ultimate result of increased number of neural progenitors, $A\beta_{1-42}$ appears to favor glial differentiation by a mechanism independent of NSC proliferation.

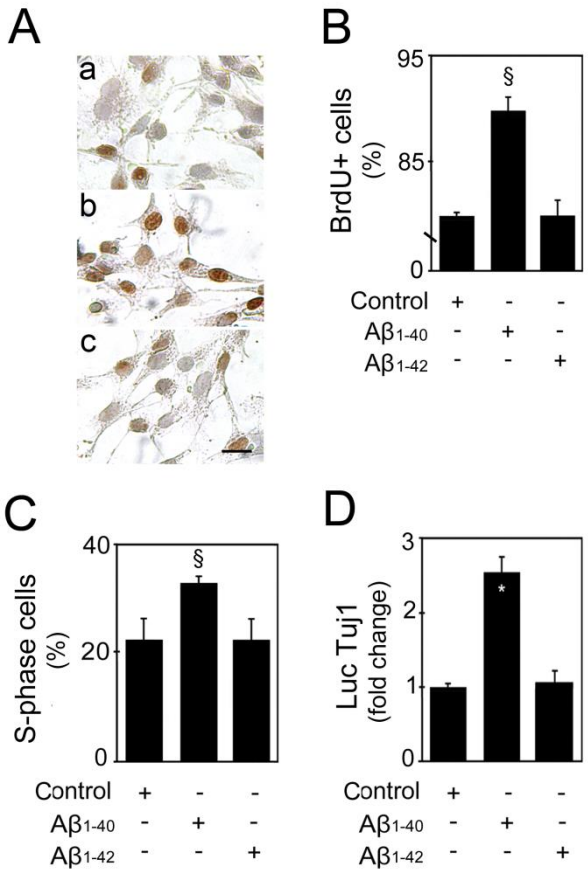


Figure 4.3. Aβ1-40 increases the S-phase of the cell cycle and neuronal-promoter activity in NSCs. *Undifferentiated cells were preincubated with $A\beta_{1-40}$ and $A\beta_{1-42}$ for 24 h and pulse-labeled for 6 h with BrdU to mark S-phase cells as described in Materials and Methods. (A) Immunostaining with rabbit anti-BrdU in control untreated cells (a), and in cells exposed to either $A\beta_{1-40}$ (b) or $A\beta_{1-42}$ (c). Scale bar, 10 µm. (B) Percentage of BrdU-positive cells analyzed by flow cytometry. (C) S-phase cells analyzed by flow cytometry. (D) Neuronal lineage-specific promoter activity. NSCs were transfected with a βIII-tubulin (Tuj1) promoter-driven luciferase construct, 24 h before $A\beta_{1-40}$ incubation. After 48 h of $A\beta_{1-40}$ treatment, luciferase activity was measured in cell lysates. $A\beta_{1-40}$ significantly increased BrdU-positive and S-phase cells as well as Tuj1 promoter activity. Results were expressed as mean ± SEM for at least three different experiments. *$p < 0.01$ and §$p < 0.05$ from controls.*

To further dissect the mechanism by which $A\beta_{1-40}$ modulates increased neuronal fate, NSCs were transfected with a βIII-tubulin (Tuj1) promoter-driven luciferase construct, 24 h before $A\beta_{1-40}$ incubation. After 48 h of $A\beta_{1-40}$ treatment, luciferase activity was measured in cell lysates. Notably, Tuj1-promoter activity was markedly induced in $A\beta_{1-40}$ treated cells, when compared with untreated NSCs ($p < 0.01$), validating data on the role of $A\beta_{1-40}$ in the neurogenesis process (Fig. 4.3D). $A\beta_{1-42}$ had no major effect on Tuj1 promoter induction. Thus, our results indicate that the pro-neurogenic effect of $A\beta_{1-40}$ results from its ability in maintaining a proliferative NSC

pool, and activating the βIII-tubulin promoter.

4.4.4. $A\beta_{1-40}$ and $A\beta_{1-42}$ increase autophagy during differentiation of NSCs

Autophagy is characterized by sequestration of cytoplasmic constituents in double or multimembrane autophagic vesicles, and their delivery to and subsequent degradation by the cell own lysosomal system (Chevallier et al., 2005; Jin et al., 2004; Wang et al., 2004; Wen et al., 2004). LC3 was originally identified as a small subunit of microtubule-associated protein 1A and 1B from rat brain (Gozuacik and Kimchi, 2004), and is usually associated to autophagosome membranes during the autophagic process (Mann and Hammarback, 1994). Therefore, LC3 has been recently proposed as a marker for autophagic vesicles and autophagic activity (Kabeya et al., 2000). A growing body of evidence points to a critical role for autophagy during proliferation and differentiation, as a response-survival mechanism to limit oxidative stress associated with these processes (Asanuma et al., 2003; Kamada et al., 2000). However, the specific role of Aβ species in mediating autophagy is largely unknown. To elucidate the role of autophagy and potential modulation by Aβ peptides in neural differentiation, we undertook various screening approaches. We investigated the LC3-I/II conversion and used the autophagic marker GFP-LC3 in differentiating NSCs undergoing differentiation after $A\beta_{1-40}$ and $A\beta_{1-42}$ treatments. Immediately after the synthesis, LC3 carboxyl terminal region is cleaved by a protease to form LC3-I peptide (18 kDa) in the cytosol (Pua et al., 2007; Zhao et al., 2010; Zhuang et al., 2011). For autophagosomal membrane localization, LC3 must be processed, by E1 and E2 enzymes, to a membrane-bound form LC3-II (16 kDa), being subsequently conjugated to the preautophagosomal membrane (Kabeya et al., 2000). Importantly, our results revealed that both $A\beta_{1-40}$ and $A\beta_{1-42}$ further increase LC3-I to -II conversion, when compared with non-treated cells (Fig. 4.4A) ($p < 0.01$). Aβ-induced autophagy during NSC differentiation was confirmed by monitoring the accumulation of LC3 by immunofluorescence. In fact, both $A\beta_{1-40}$ and $A\beta_{1-42}$ increased GFP-LC3 puncta in NSCs undergoing differentiation (Fig. 4.4B). Moreover, the autophagy-related protein 9 (Atg9), a multi-pass membrane protein that localizes to the pre-autophagosomal structure, markedly augmented throughout differentiation in cells treated with $A\beta_{1-40}$ or $A\beta_{1-42}$ ($p < 0.01$) (Fig. r.4C).

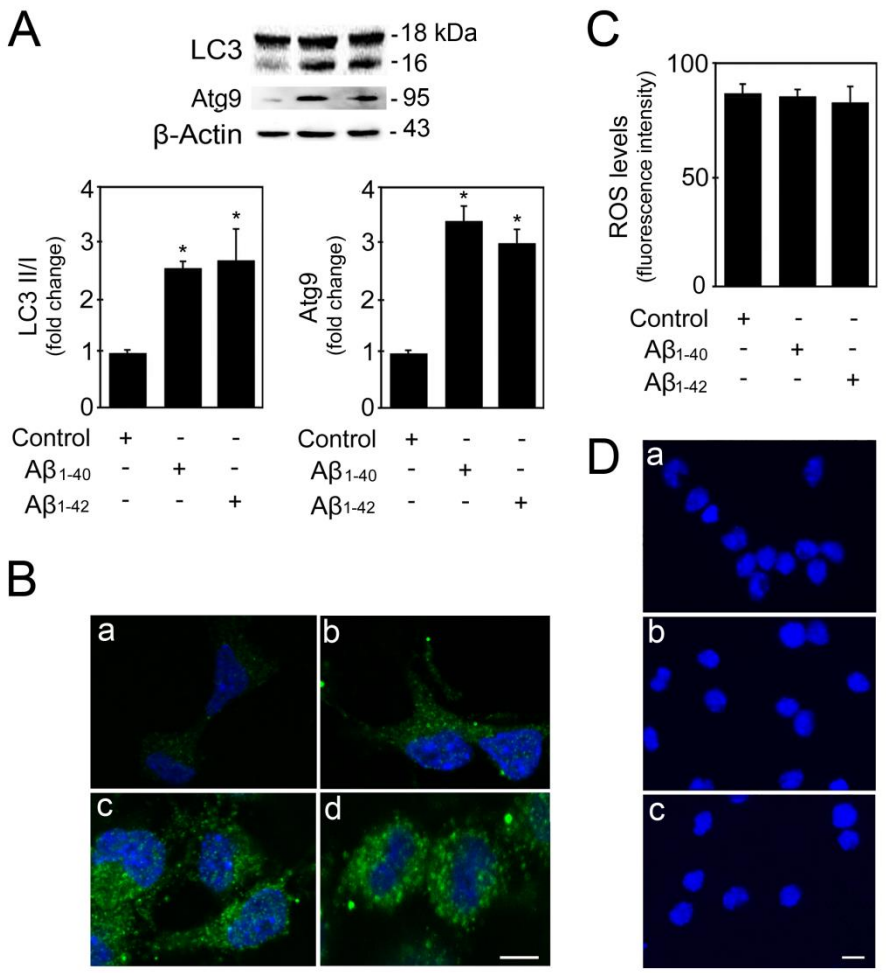


Figure 4.4. Autophagy is increased in Aβ1-40- and Aβ1-42-treated NSCs. *Cells were induced to differentiate, treated with either $A\beta_{1-40}$ or $A\beta_{1-42}$ for 24 h, and then collected for Western blot, apoptosis, confocal microscopy and flow cytometry analyses as described in Material and Methods. (A) Representative immunoblots of LC3-I, LC3-II and Atg9 (top) and respective protein levels (bottom). β-Actin was used as loading control. (B) Representative confocal images of GFP-LC3 puncta in undifferentiated (a) or differentiated (b) conditions, and treated with $A\beta_{1-40}$ (c) or $A\beta_{1-42}$ (d). Scale bar, 5 µm. (C) Intracellular ROS quantification of fluorescent DCF by flow cytometry. (D) Representative images of Hoechst staining in control cells (a), or cells treated with $A\beta_{1-40}$ (b) or $A\beta_{1-42}$ (c). Scale bar, 10 µm. Autophagy is increased after Aβ treatment; however, ROS levels and apoptosis were not altered. Results were expressed as mean ± SEM for at least three different experiments. *p < 0.01 from controls.*

Finally, in the concentrations used, Aβ peptides did not increase redox status (Fig. 4.4C) nor induced cell death throughout NSC differentiation, as detected by Hoechst (Fig. 4.4D) and PI and Annexin-V staining. These data suggest that the protective mechanism of autophagy is potentially modulated by Aβ peptides to promote neural differentiation.

4.4.5. Inhibition of autophagy reverts the effects of $A\beta_{1-40}$ and $A\beta_{1-42}$ in enhancing lineage-specific markers in NSCs

To clarify the contribution of autophagy in Aβ-regulated NSC fate, we tested the effects of autophagy inhibition on the ability of both $A\beta_{1-40}$ and $A\beta_{1-42}$ to increase lineage-specific markers. We used the autophagy inhibitor 3-MA and re-evaluated the effects of $A\beta_{1-40}$ and $A\beta_{1-42}$ in neuronal and astrocytic differentiation, respectively. Confocal microscopy confirmed that treatment of NSCs with 3-MA markedly decreased the number of GFP-LC3-labeled puncta (Fig. 4.5A). Similar results were obtained after treatment with a second autophagy inhibitor, wortmannin (data not shown). Cell death increased in the presence of 3-MA in both Aβ-treated and untreated cells, as determined by the LDH assay (Fig. 4.5B) ($p < 0.01$) and PI and Annexin-V staining (data not shown). In addition, flow cytometry analysis revealed that the effects of $A\beta_{1-40}$ and $A\beta_{1-42}$ in NSC fate were completely reverted after incubation with 3-MA. Notably, the inhibition of autophagy diminished the number of βIII-tubulin- and GFAP-positive cells in NSCs incubated with $A\beta_{1-40}$ and $A\beta_{1-42}$, when compared with control (Fig. 4.5C) ($p < 0.01$). Finally, immunocytochemistry analysis of both βIII-tubulin and GFAP confirmed the inhibitory effects of 3-MA in $A\beta_{1-40}$ and $A\beta_{1-42}$-induced neuronal and astrocytic-specific protein markers, respectively (Fig. 4.5D). These results indicate that $A\beta_{1-40}$ and $A\beta_{1-42}$ may be important regulators of neural differentiation through an autophagy-dependent manner.

4.5. Discussion

Aβ peptides have important physiological functions before they aggregate and form amyloid fibrils (Tanida et al., 2002; Tanida et al., 2001). However, our understanding of Aβ signals controlling these processes is limited. In this study, we demonstrate that active fragments of $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides, but not $A\beta_{25-35}$ peptide, significantly stimulate neural-lineage in NSCs, through a mechanism at least in part dependent on autophagy signaling mechanisms.

Much attention has been centered on toxic effects of Aβ peptides (Parihar and Brewer, 2010; Pearson and Peers, 2006; Plant et al., 2003; Ramsden et al., 2002). In fact, Aβ peptides are strongly implicated in AD, promoting synaptic dysfunction and neuronal cell death by a mechanism involving increased oxidative stress and disruption of cellular calcium homeostasis (Klein, 2002; Pike et al., 1993). However, Aβ peptides may also activate kinase enzymes, protect against oxidative stress (Mattson, 1997),

regulate cholesterol transport and function as transcription factors (Baruch-Suchodolsky and Fischer, 2009).

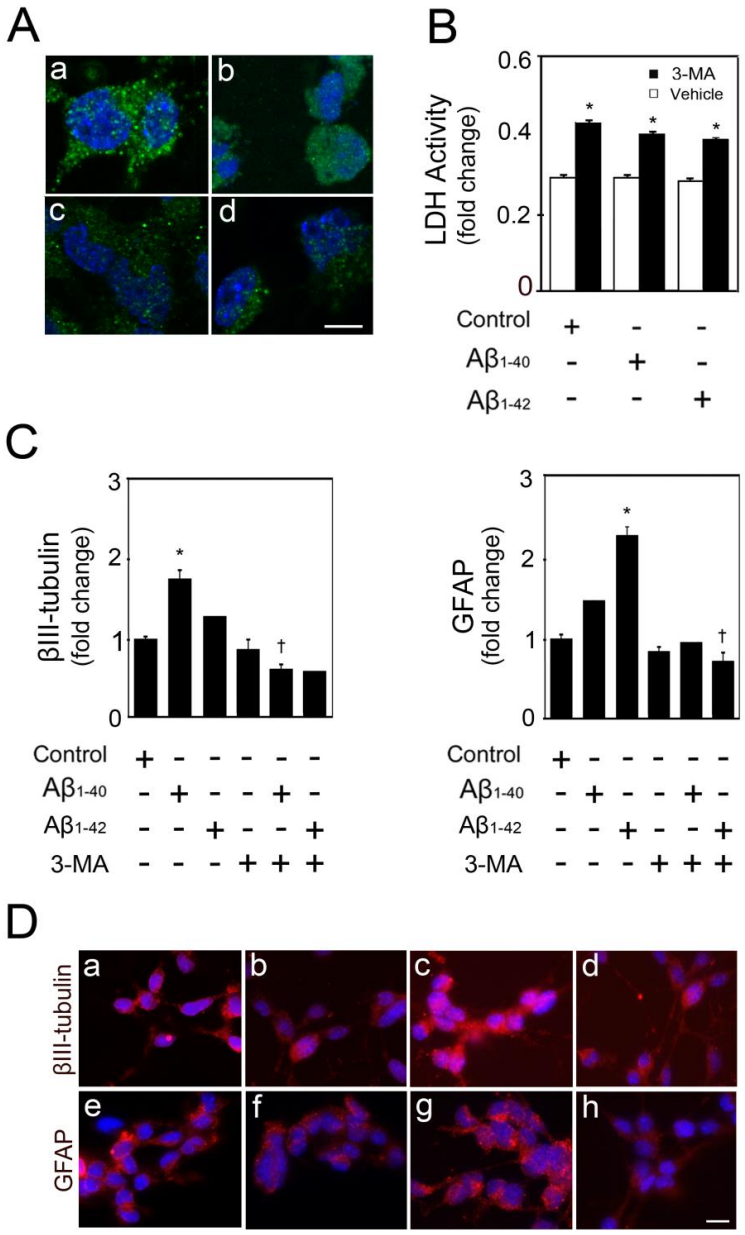


Figure 4.5. The autophagy inhibitor 3-MA abrogated the effects of Aβ1-40 and Aβ1-42 in neuronal and astrocytic differentiation of NSCs. *Mouse NSCs were induced to differentiate and treated with either 3-MA (5 mM), $A\beta_{1-40}$ or $A\beta_{1-42}$ for 24 h. Cells were collected for LDH, flow citometry, confocal and immunocytochemistry analysis as described in Material and Methods. (A) Representative confocal images of GFP-LC3 puncta in control cells (a), or in cells treated with either 3-MA (b), $A\beta_{1-40}$ + 3-MA (c), $A\beta_{1-42}$ + 3-MA (d) for 24 h. Scale bar, 5 μm. (B) LDH release. (C) Percentage of βIII-tubulin- and GFAP-positive assessed by flow cytometry analysis. (D) Representative images of βIII-tubulin- and GFAP-positive cells in controls (a and e), and in cells treated with either 3-MA (b and f), $A\beta_{1-40}$ + 3-MA (c and g), $A\beta_{1-42}$ + 3-MA (d and h) for 24 h. Results were expressed as mean ± SEM for at least three different experiments. *p < 0.01 from controls; †p < 0.01 from Aβ alone.*

The beneficial role for $A\beta_{1-40}$ peptide in neurons has previously been reported *in vivo,* where $A\beta_{1-40}$ was shown to inhibit amyloid deposition (Chen and Dong, 2009). Here, we first determined the effects of $A\beta_{1-40}$, $A\beta_{1-42}$ and $A\beta_{25-35}$ in expression levels of neural markers during differentiation of mouse NSC. Soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ increased both βIII-tubulin and GFAP levels; however, $A\beta_{1-40}$ was preferentially pro-neurogenic, while $A\beta_{1-42}$ was pro-astrocytic. In addition, we have shown that $A\beta_{1-40}$ not only promotes NSC self-renewal but also enhances the activity of neuronal differentiation. In fact, after $A\beta_{1-40}$ treatment of NSCs, the S-phase of the cell cycle and BrdU labeling were markedly enhanced. A potential direct effect of $A\beta_{1-40}$ on βIII-tubulin promoter activity and its influence on neurogenesis may be further clarified in knockdown experiments of βIII-tubulin after $A\beta_{1-40}$ treatment.

Our findings are in accordance with a previous report showing that non-aggregated $A\beta_{1-40}$ and $A\beta_{1-42}$ promote neuronal and astrocytic cell fate in neural progenitor cells (Kim et al., 2007). However, in that study, $A\beta_{1-42}$ also increases NSC proliferation, while we did not observed significant alterations in self-renewal after $A\beta_{1-42}$ incubation. Distinct cellular models and experimental conditions in both studies could account for this apparently discrepant result. The authors have used NSCs derived from 18-dpc rat fetal brain, while we used NSC derived from 14.5-dpc mouse fetal brain. Moreover, others have demonstrated a predominant effect of $A\beta_{1-42}$ in neurogenesis (Chen and Dong, 2009). In fact, the number of βIII-tubulin-positive neurons was increased by $A\beta_{1-42}$ in one study (Calafiore et al., 2012; Heo et al., 2007; Lopez-Toledano and Shelanski, 2004), while others reported that low concentrations of oligomeric $A\beta_{1-42}$ peptides enhance proliferation and neuronal differentiation of NSCs (Lopez-Toledano and Shelanski, 2004). Hence, although the absence of $A\beta_{25-35}$ effect in eliciting either self-renewal or neural differentiation appears to be consensual in the literature, the debate is still ongoing as to the role of different forms of Aβ on the neurogenesis process.

On the other hand, studies using animal models of AD have proposed the opposite effect of Aβ peptides in neurogenesis (Heo et al., 2007). However, these results appear to be correlated with the decreased levels of soluble Aβ peptide observed in AD. In fact, Aβ peptide has already been reported to have differential effects on human embryonic stem cell proliferation depending on its aggregation level (Haughey et al., 2002a; Haughey et al., 2002b); the neurogenic functions of Aβ were greatly

reduced when this active peptide was in aggregated forms. Consistent with this, others have demonstrated that higher levels of Aβ monomers, oligomers, and fibrils in the brain microenvironment in individuals with amyloidosis-β induce oxidative stress and have a negative effect on endogenous neurogenesis (Porayette et al., 2009). AD is a dynamic disease process, and it is plausible that an increase in neuronal differentiation is followed by an overall decline as Aβ aggregates and loses its neurogenic functions.

By evaluating the lipidated LC3-II and Atg9 markers of autophagy, our results have also shown that low μM levels of soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ markedly increase autophagy throughout NSC differentiation. In fact, autophagy has been shown to play a critical role in stem cell maintenance and in a variety of cell differentiation processes (Mazur-Kolecka et al., 2006). Recently, it has been revealed that NSCs may activate autophagy to also fulfill their high energy demands (Asanuma et al., 2003; Vessoni et al., 2012; Zhao et al., 2010). The link between Aβ peptides and autophagy has also previously been described. Autophagy protects neurons from Aβ-induced toxicity (Vázquez et al., 2012) and serves as a mechanism that mediates clearance of ubiquitinated Aβ to clear molecular debris and restore neurotransmitter balance (Hung et al., 2009). Nevertheless, the majority of studies relating Aβ peptide with autophagy have demonstrated that Aβ peptide triggers autophagic cell death by inducing ROS accumulation (Khandelwal et al., 2011; Zheng et al., 2006a). However, although ROS play an important role in the induction of autophagy (Lipinski et al., 2010; Wang et al., 2010; Yang et al., 2012), our results showed that autophagy induced by Aβ peptides during neural differentiation was not associated with increased levels of oxidative stress, and other Aβ-mediated events may account for this cellular outcome. In addition, the autophagy inhibitor 3-MA significantly increased cell death, indicating that autophagy triggered by soluble low levels of Aβ might operate as a NSC survival response. Indeed, autophagy was shown to inhibit apoptosis by several mechanisms (Gurusamy and Das, 2009). Remarkably, 3-MA inhibited Aβ-mediated effects throughout differentiation, showing that neurogenesis and gliogenesis are favored in $A\beta_{1-40}$- and $A\beta_{1-42}$-treated NSC as the result of increased autophagy activity. It would be interesting to dissect the molecular mechanisms by which soluble Aβ species promote neural differentiation, and to further understand how Aβ may induce autophagy through a ROS-independent manner. In fact, Aβ peptide was already found in the nucleus to activate p53 promoter activity (Yang and Klionsky, 2010), possibly directly regulating the expression of many other genes.

Together, our results corroborate the beneficial role of soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ in mediating neurogenesis and astrogliogenesis, respectively, and identify autophagy as an essential mechanism of Aβ-induced neural differentiation.

Acknowledgments

We thank Dr. Fred Gage (The Salk Institute, California, USA) for generously supplying the luciferase reporter constructs and Dr. Tamotsu Yoshimori (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) for the pGFP-LC3 construct. This work was supported by grants PTDC/SAU-NMC/117877/2010 and Pest-OE/SAU/UI4013/2011, and fellowships SFRH/BD/43959/2008 (M.B.F.) and SFRH/BD/68368/2010 (J.M.X.) from Fundação para a Ciência e a Tecnologia, Portugal.

5. General Discussion and Concluding Remarks

The studies presented in this thesis contribute to a better understanding of the apoptotic mechanisms implicated in AD and extend our knowledge on the role of apoptosis-related molecules and Aβ peptides as components of neural proliferation and differentiation processes.

In brief, we demonstrated a role for p63 during Aβ-induced apoptosis and the existence of a c-Jun-dependent mechanism regulating the abundance of anti-apoptotic ΔNp63 in the context of Aβ-induced stress. Importantly, the anti-apoptotic bile acid TUDCA was efficient in partially modulating these effects. Next, we implicated p63 in regulating neural differentiation by demonstrating that the TAp63γ isoform cross-talks with key regulators of neurogenesis, specifically with the histone H3 lysine 27-specific demethylase JMJD3. Finally, we explored the effect of soluble Aβ fragments in modulating NSC fate, and identified autophagy as an essential mechanism of Aβ-induced neural differentiation (Fig. 5.1). The data presented here provide new insights into the regulation of Aβ-induced cell death and neural differentiation by apoptosis-associated molecules, and represents a step forward in understanding the molecular mechanisms by which Aβ peptides mediate neural differentiation. In this last concluding chapter, we integrate our findings, and address future perspectives arising from these studies.

The p63 protein is a multi-isoform p53 family member that is involved in cell death (Gressner et al., 2005). In the present work, we evaluated the effect of Aβ-induced apoptosis in the regulation of p63 abundance. Aβ treatment of PC12 neuronal-like cells and rat cortical neurons caused an upregulation of the pro-apoptotic isoform TAp63γ and downregulation of the anti-apoptotic isoform ΔNp63γ, which allowed the manifestation of apoptosis. In parallel, we showed that genotoxic drugs such as doxorubicin and cisplatin increased TAp63γ levels, concurrent with the reduction of ΔNp63γ abundance in PC12 cells. In fact, it is well documented that genotoxic signals regulate p63 protein levels, promoting phosphorylation-induced, proteasome-mediated degradation of the pro-survival isoform ΔNp63 (Chatterjee et al., 2008; Lazzari et al., 2011). However, this evidence was only reported for α isoforms. Importantly, the bile acid TUDCA, a well characterized endogenous potent inhibitor of apoptosis in different cell types (Ramalho et al., 2004; Solá et al., 2006; Viana et al., 2010), strongly modulated Aβ-induced apoptosis, while preventing the changes in p63 levels, further suggesting that the reduction of ΔNp63 levels is important for apoptosis to occur.

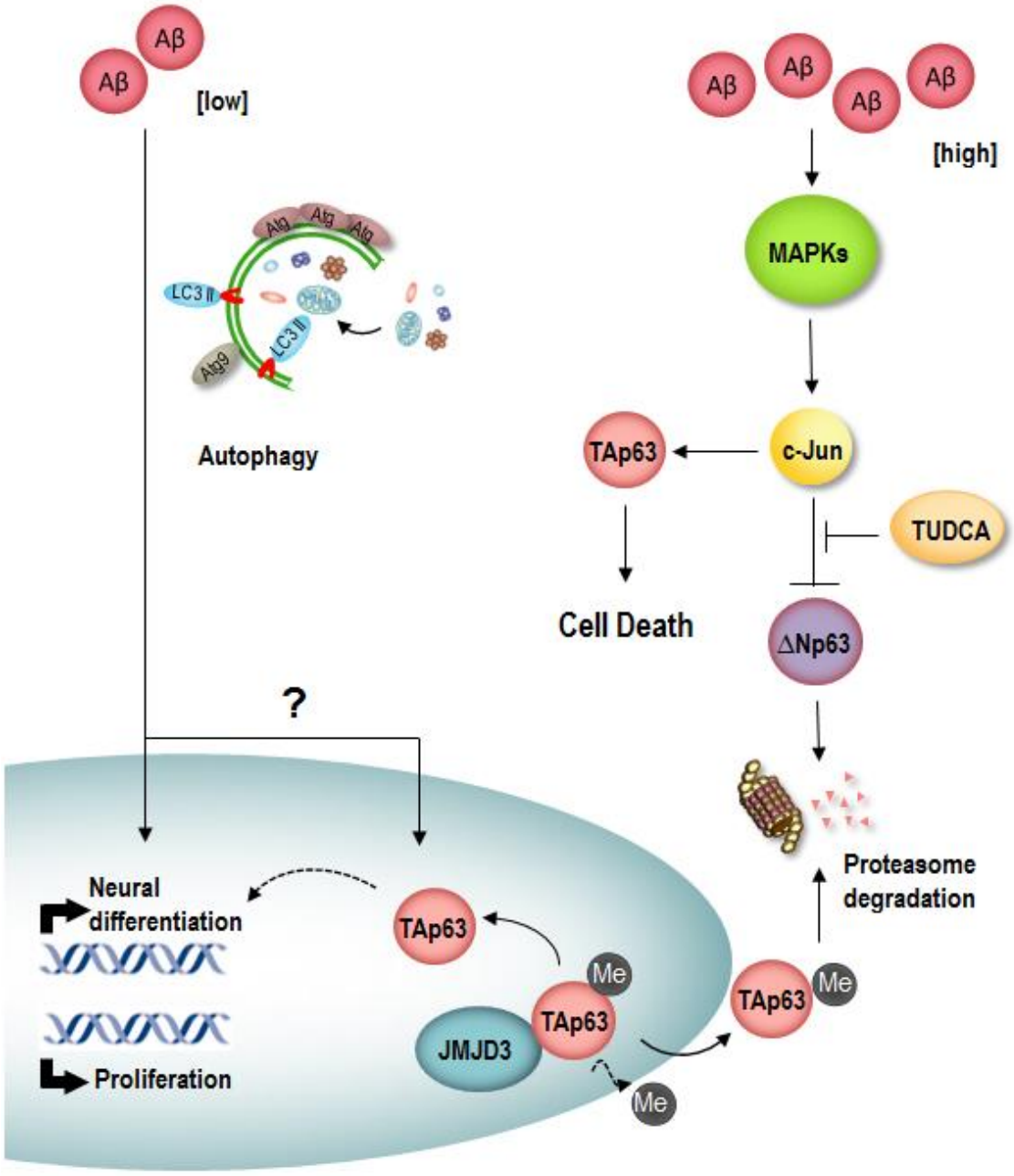


Figure 5.1. Graphical abstract. *In the context of Aβ-induced stress, the abundance of ΔNp63 and TAp63 is regulated by a c-Jun-dependent mechanism. Importantly, the bile acid TUDCA partially modulates these effects. Aβ-mediated reduction of ΔNp63 protein levels may be influenced by the MAPK pathway and occurs in a proteasome-dependent manner. The TAp63γ isoform cross-talks with key regulators of neurogenesis, namely the histone demethylase JMJD3, to redirect stem cells to differentiation, as an alternative to cell death. Finally, low levels of soluble Aβ peptides may also modulate neural differentiation. Autophagy appears to be an essential player in Aβ-induced neural differentiation. Interactions that were suggested or shown indirectly are represented by dashed lines.*

c-Jun, a key regulator of stress-mediated cell death has been shown to play a role in Aβ-induced toxicity (Estus et al., 1997; Viana et al., 2010). In addition, it has been

reported that this member of the AP-1 family of transcription factors modulates the abundance of TAp63 (Yao et al., 2010b). Indeed, here we demonstrated that ΔNp63 reduction was associated with increased c-Jun protein levels after Aβ treatment in PC12 neuronal-like cells and cortical neurons. Further, Aβ-mediated reduction of ΔNp63 protein levels appeared to be influenced by the MAPK pathway and occur in a proteasome-dependent manner. In fact, it has been reported that Aβ may inhibit the proteasome (Gregori et al., 1995; Tseng et al., 2008). Nevertheless, our results are in accordance with other studies demonstrating that important mediators of AD are degraded in a proteasome-dependent fashion (Lanni et al., 2010; Roselli et al., 2005), thus contributing to AD pathogenesis. Therefore, the use of distinct cell model systems and Aβ exposure conditions may help reconcile data from different studies. Of note, while TUDCA rescued Aβ-induced decrease of ΔNp63 levels, it also prevented the increase in c-Jun levels promoted by the peptide. Together, these findings demonstrate that the abundance of ΔNp63 and TAp63 in response to Aβ-induced cell stress is coordinately regulated by a c-Jun-dependent mechanism, and highlight the importance of discovering novel targets for potential therapeutic intervention. Since TUDCA is a cholesterol-derived molecule and presents high affinity to lipid membranes (Solá et al., 2006), it would be interesting to further explore in the future, whether this bile acid may also interfere with Aβ load (Nunes et al., 2012) and entrance in the cells.

Curiously, it has been recently suggested that molecules involved in the core of the executing apoptosis machinery play a dual role in differentiation and cell death. We have previously demonstrated the involvement of specific apoptosis-related miRNAs and proteins, including p53, caspases and calpains, in mouse NSC differentiation, and also showed that p53 interacts with key regulators of neurogenesis, such as JMJD3, to redirect stem cells to differentiation, as an alternative to cell death (Aranha et al., 2009; Santos et al., 2012b; Solá et al., 2011; Solá et al., 2012). Given the functional and structural similarities of p63 and p53, we next investigated the role of p63 during mouse NSC differentiation and evaluated whether p63 interacts with JMJD3 in this cellular context. In fact, in contrast with p53 and p73, there is still a breach in the literature regarding p63 potential involvement in neurogenesis by cell death-independent mechanisms. In spite of other studies claiming that p63 is dispensable for neurogenesis, p63 was shown to be expressed in neurogenic niches, such as the SVZ of the lateral ventricle in postnatal mice, supporting the idea that p63 may have a key role during neurogenesis (Hernández-Acosta et al., 2011). Our results reveal for the first time a role

for p63 in neural progenitors, directing NSCs to a neuronal phenotype. In fact, our data support the hypothesis that JMJD3-induced TAp63γ nuclear translocation might direct NSCs to a neuronal fate specification, through the transcriptional activation of pro-neuronal genes. Overexpression of TAp63γ during early differentiation of NSCs resulted in increased β-III tubulin levels, whereas downregulation of TAp63γ expression by specific p63 siRNA abrogated the increase in β-III tubulin. Interestingly, we showed that JMJD3 directly interacts with p63 to induce p63 demethylation and subsequent p63 stabilization during mouse neural differentiation. A deepest knowledge of the regulatory modifications of p63 protein will certainly improve our understanding of p63 function during neural differentiation. In this respect, future studies are warranted to determine the specific p63 residues demethylated by JMJD3 and to address the importance of TAp63γ demethylation in neurogenesis by modification of the identified residues. On the other hand, since TUDCA modulates p63 during apoptosis, it would be interesting to also clarify whether this bile acid regulates p63 in this cellular context, also affecting NSC fate.

At last, we investigated whether low levels of soluble Aβ peptides may also modulate neural differentiation. In fact, several studies have shown that classical triggers of apoptosis may positively influence neural differentiation (Solá et al., 2012). In addition, Aβ species are highly multifunctional peptides with significant non-pathological activities. However, the precise molecular pathways underlying the regulation of NSC proliferation and differentiation by different Aβ peptides are still not fully defined. Our results demonstrate that $A\beta_{1-40}$ promotes NSC self-renewal and enhances mostly neuronal differentiation. In fact, after $A\beta_{1-40}$ treatment of NSCs, S-phase of the cell cycle, BrdU labeling, and βIII-tubulin promoter-driven luciferase activity were markedly enhanced. In contrast, $A\beta_{1-42}$ promoted mostly astrocytic cell fate in NSCs, having no effect in NSC proliferation. These results are in part in discordance with other previous reports (Chen and Dong, 2009; Heo et al., 2007; López-Toledano and Shelanski, 2004). Indeed, there is variance in the literature regarding the experimental conditions used to address the neurogenic effects of Aβ, which can apparently explain these discrepancies in the results.

Due to the involvement of autophagy in cellular survival and differentiation, development and homeostasis (Aburto et al., 2012; Levine and Kroemer, 2008; Mizushima and Levine, 2010; Vessoni et al., 2012), and since autophagy is involved in Aβ-mediated effects (Hung et al., 2009), we explored the role of autophagy in Aβ-

induced alterations of neural fate. In fact, the correlation between pro-neurogenic effects of Aβ peptides and autophagy has not been explored before. Our results demonstrated that low levels of soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ markedly increase autophagy throughout NSC differentiation. Surprisingly, neurogenesis and gliogenesis were favored in $A\beta_{1-40}$- and $A\beta_{1-42}$-treated NSCs as the result of enhanced autophagy activity, without increased levels of oxidative stress and apoptosis. The inhibition of autophagy in this cellular context significantly increased cell death, indicating that autophagy triggered by soluble low levels of Aβ might operate as a NSC survival response.

As a final note, it is important to look beyond the role Aβ peptides during neural differentiation and to further dissect the precise molecular targets of soluble Aβ species during this process. Taking into account the role of apoptosis-related proteins during neural differentiation, namely the p63 protein, it is tempting to think that Aβ-induced effect on NSCs might be partially dependent on p63 and c-Jun. Finally, it would be interesting to explore how low levels of Aβ regulate p63 expression in neural differentiation; how Aβ may induce autophagy through a ROS-independent manner; and whether TUDCA regulates the survival process of autophagy in both apoptotic and differentiation contexts.

The poor survival and differentiation levels of stem cells after either transplantation or neural injury have been one of the major problems of stem cell-based therapy. With the growing identification of the regulatory networks between neural apoptosis and differentiation, it is likely that we will witness relevant advances in potential therapeutic tools that contribute to a more efficient use of stem cells in neural-replacement therapies.

References

Aburto, M. R., Sánchez-Calderón, H., Hurlé, J. M., Varela-Nieto, I. and Magariños, M. (2012). Early otic development depends on autophagy for apoptotic cell clearance and neural differentiation. *Cell Death Dis* 3, e394.

Agami, R. and Bernards, R. (2000). Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. *Cell* 102, 55-66.

Agostini, M., Tucci, P., Chen, H., Knight, R. A., Bano, D., Nicotera, P., McKeon, F. and Melino, G. (2010). p73 regulates maintenance of neural stem cell. *Biochem Biophys Res Commun* 403, 13-7.

Agostini, M., Tucci, P., Killick, R., Candi, E., Sayan, B. S., Rivetti di Val Cervo, P., Nicotera, P., McKeon, F., Knight, R. A., Mak, T. W. et al. (2011a). Neuronal differentiation by TAp73 is mediated by microRNA-34a regulation of synaptic protein targets. *Proc Natl Acad Sci U S A* 108, 21093-8.

Agostini, M., Tucci, P., Steinert, J. R., Shalom-Feuerstein, R., Rouleau, M., Aberdam, D., Forsythe, I. D., Young, K. W., Ventura, A., Concepcion, C. P. et al. (2011b). microRNA-34a regulates neurite outgrowth, spinal morphology, and function. *Proc Natl Acad Sci U S A* 108, 21099-104.

Aloyz, R. S., Bamji, S. X., Pozniak, C. D., Toma, J. G., Atwal, J., Kaplan, D. R. and Miller, F. D. (1998). p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. *J Cell Biol* 143, 1691-703.

Altman, J. (1969). Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. *J Comp Neurol* 137, 433-57.

Altman, J. and Das, G. D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Comp Neurol* 124, 319-35.

Alvarez, A. R., Sandoval, P. C., Leal, N. R., Castro, P. U. and Kosik, K. S. (2004). Activation of the neuronal c-Abl tyrosine kinase by amyloid-beta-peptide and reactive oxygen species. *Neurobiol Dis* 17, 326-36.

Alvarez-Buylla, A. and Garcia-Verdugo, J. M. (2002). Neurogenesis in adult subventricular zone. *J Neurosci* 22, 629-34.

Anderson, A. J., Cummings, B. J. and Cotman, C. W. (1994). Increased immunoreactivity for Jun- and Fos-related proteins in Alzheimer's disease: association with pathology. *Exp Neurol* 125, 286-95.

Anderson, A. J., Su, J. H. and Cotman, C. W. (1996). DNA damage and apoptosis in Alzheimer's disease: colocalization with c-Jun immunoreactivity, relationship to brain area, and effect of postmortem delay. *J Neurosci* 16, 1710-9.

Anderson, C. N. and Tolkovsky, A. M. (1999). A role for MAPK/ERK in sympathetic neuron survival: protection against a p53-dependent, JNK-independent induction of apoptosis by cytosine arabinoside. *J Neurosci* 19, 664-73.

Angel, P. and Karin, M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta* 1072, 129-57.

Aranha, M. M., Santos, D. M., Solá, S., Steer, C. J. and Rodrigues, C. M. (2011). miR-34a regulates mouse neural stem cell differentiation. *PLoS One* 6, e21396.

Aranha, M. M., Santos, D. M., Xavier, J. M., Low, W. C., Steer, C. J., Solá, S. and Rodrigues, C. M. (2010). Apoptosis-associated microRNAs are modulated in mouse, rat and human neural differentiation. *BMC Genomics* 11, 514.

Aranha, M. M., Solá, S., Low, W. C., Steer, C. J. and Rodrigues, C. M. (2009). Caspases and p53 modulate FOXO3A/Id1 signaling during mouse neural stem cell differentiation. *J Cell Biochem* 107, 748-58.

Arico, S., Petiot, A., Bauvy, C., Dubbelhuis, P. F., Meijer, A. J., Codogno, P. and Ogier-Denis, E. (2001). The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. *J Biol Chem* 276, 35243-6.

Armesilla-Diaz, A., Bragado, P., Del Valle, I., Cuevas, E., Lazaro, I., Martin, C., Cigudosa, J. C. and Silva, A. (2009). p53 regulates the self-renewal and differentiation of neural precursors. *Neuroscience* 158, 1378-89.

Armstrong, J. F., Kaufman, M. H., Harrison, D. J. and Clarke, A. R. (1995). High-frequency developmental abnormalities in p53-deficient mice. *Curr Biol* 5, 931-6.

Asanuma, K., Tanida, I., Shirato, I., Ueno, T., Takahara, H., Nishitani, T., Kominami, E. and Tomino, Y. (2003). MAP-LC3, a promising autophagosomal marker, is processed during the differentiation and recovery of podocytes from PAN nephrosis. *FASEB J* 17, 1165-7.

Atwood, C. S., Obrenovich, M. E., Liu, T., Chan, H., Perry, G., Smith, M. A. and Martins, R. N. (2003). Amyloid-beta: a chameleon walking in two worlds: a review of the trophic and toxic properties of amyloid-beta. *Brain Res Brain Res Rev* 43, 1-16.

Aydin, D., Weyer, S. W. and Müller, U. C. (2012). Functions of the APP gene family in the nervous system: insights from mouse models. *Exp Brain Res* 217, 423-34.

Bakkers, J., Camacho-Carvajal, M., Nowak, M., Kramer, C., Danger, B. and Hammerschmidt, M. (2005). Destabilization of DeltaNp63alpha by Nedd4-mediated ubiquitination and Ubc9-mediated sumoylation, and its implications on dorsoventral patterning of the zebrafish embryo. *Cell cycle* 4, 790-800.

Balietti, M., Tamagnini, F., Fattoretti, P., Burattini, C., Casoli, T., Platano, D., Lattanzio, F. and Aicardi, G. (2012). Impairments of synaptic plasticity in aged animals and in animal models of Alzheimer's disease. *Rejuvenation Res* 15, 235-8.

Baruch-Suchodolsky, R. and Fischer, B. (2009). Abeta40, either soluble or aggregated, is a remarkably potent antioxidant in cell-free oxidative systems. *Biochemistry* 48, 4354-70.

Behl, C., Davis, J. B., Lesley, R. and Schubert, D. (1994). Hydrogen peroxide mediates amyloid beta protein toxicity. *Cell* 77, 817-27.

Behrens, A., Sibilia, M. and Wagner, E. F. (1999). Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nature genetics* 21, 326-9.

Benchimol, S. (2004). p53--an examination of sibling support in apoptosis control. *Cancer Cell* 6, 3-4.

Bentahir, M., Nyabi, O., Verhamme, J., Tolia, A., Horré, K., Wiltfang, J., Esselmann, H. and De Strooper, B. (2006). Presenilin clinical mutations can affect gamma-secretase activity by different mechanisms. *J Neurochem* 96, 732-42.

Bernal, G. M. and Peterson, D. A. (2004). Neural stem cells as therapeutic agents for age-related brain repair. *Aging Cell* 3, 345-51.

Bernier, P. J. and Parent, A. (1998). Bcl-2 protein as a marker of neuronal immaturity in postnatal primate brain. *J Neurosci* 18, 2486-97.

Biebl, M., Cooper, C. M., Winkler, J. and Kuhn, H. G. (2000). Analysis of neurogenesis and programmed cell death reveals a self-renewing capacity in the adult rat brain. *Neurosci Lett* 291, 17-20.

Billon, N., Terrinoni, A., Jolicoeur, C., McCarthy, A., Richardson, W. D., Melino, G. and Raff, M. (2004). Roles for p53 and p73 during oligodendrocyte development. *Development* 131, 1211-20.

Blass, J. P. and Gibson, G. E. (2006). Correlations of disability and biologic alterations in Alzheimer brain and test of significance by a therapeutic trial in humans. *J Alzheimers Dis* 9, 207-18.

Blennow, K. and Zetterberg, H. (2009). Cerebrospinal fluid biomarkers for Alzheimer's disease. *J Alzheimers Dis* 18, 413-7.

Blurton-Jones, M. and Laferla, F. M. (2006). Pathways by which Abeta facilitates tau pathology. *Curr Alzheimer Res* 3, 437-48.

Boland, B., Kumar, A., Lee, S., Platt, F. M., Wegiel, J., Yu, W. H. and Nixon, R. A. (2008). Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. *J Neurosci* 28, 6926-37.

Bonaguidi, M. A., Wheeler, M. A., Shapiro, J. S., Stadel, R. P., Sun, G. J., Ming, G. L. and Song, H. (2011). In vivo clonal analysis reveals self-renewing and multipotent adult neural stem cell characteristics. *Cell* 145, 1142-55.

Bourdon, J. C. (2007). p53 Family isoforms. *Curr Pharm Biotechnol* 8, 332-6.

Bradham, C. A., Qian, T., Streetz, K., Trautwein, C., Brenner, D. A. and Lemasters, J. J. (1998). The mitochondrial permeability transition is required for tumor necrosis factor alpha-mediated apoptosis and cytochrome c release. *Mol Cell Biol* 18, 6353-64.

Brooks, C. L. and Gu, W. (2003). Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. *Curr Opin Cell Biol* 15, 164-71.

Browes, C., Rowe, J., Brown, A. and Montano, X. (2001). Analysis of trk A and p53 association. *FEBS Lett* 497, 20-5.

Bruckner, S. R., Tammariello, S. P., Kuan, C. Y., Flavell, R. A., Rakic, P. and Estus, S. (2001). JNK3 contributes to c-Jun activation and apoptosis but not oxidative stress in nerve growth factor-deprived sympathetic neurons. *J Neurochem* 78, 298-303.

Brynczka, C. and Merrick, B. A. (2007). Nerve growth factor potentiates p53 DNA binding but inhibits nitric oxide-induced apoptosis in neuronal PC12 cells. *Neurochem Res* 32, 1573-85.

Brüstle, O., Spiro, A. C., Karram, K., Choudhary, K., Okabe, S. and McKay, R. D. (1997). In vitro-generated neural precursors participate in mammalian brain development. *Proc Natl Acad Sci U S A* 94, 14809-14.

Bunk, E. C., König, H. G., Bernas, T., Engel, T., Henshall, D. C., Kirby, B. P. and Prehn, J. H. (2010). BH3-only proteins BIM and PUMA in the regulation of survival and neuronal differentiation of newly generated cells in the adult mouse hippocampus. *Cell Death Dis* 1, e15.

Burgold, T., Spreafico, F., De Santa, F., Totaro, M. G., Prosperini, E., Natoli, G. and Testa, G. (2008). The histone H3 lysine 27-specific demethylase Jmjd3 is required for neural commitment. *PLoS ONE* 3, e3034.

Calafiore, M., Battaglia, G., Zappalà, A., Trovato-Salinaro, E., Caraci, F., Caruso, M., Vancheri, C., Sortino, M. A., Nicoletti, F. and Copani, A. (2006). Progenitor cells from the adult mouse brain acquire a neuronal phenotype in response to beta-amyloid. *Neurobiol Aging* 27, 606-13.

Calafiore, M., Copani, A. and Deng, W. (2012). DNA polymerase-β mediates the neurogenic effect of β-amyloid protein in cultured subventricular zone neurospheres. *J Neurosci Res* 90, 559-67.

Cameron, H. A., Woolley, C. S., McEwen, B. S. and Gould, E. (1993). Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. *Neuroscience* 56, 337-44.

Campbell, D. S. and Holt, C. E. (2003). Apoptotic pathway and MAPKs differentially regulate chemotropic responses of retinal growth cones. *Neuron* 37, 939-52.

Cancino, G. I., Toledo, E. M., Leal, N. R., Hernandez, D. E., Yévenes, L. F., Inestrosa, N. C. and Alvarez, A. R. (2008). STI571 prevents apoptosis, tau phosphorylation and behavioural impairments induced by Alzheimer's beta-amyloid deposits. *Brain* 131, 2425-42.

Capsoni, S., Ugolini, G., Comparini, A., Ruberti, F., Berardi, N. and Cattaneo, A. (2000). Alzheimer-like neurodegeneration in aged antinerve growth factor transgenic mice. *Proc Natl Acad Sci U S A* 97, 6826-31.

Caricasole, A., Copani, A., Caraci, F., Aronica, E., Rozemuller, A. J., Caruso, A., Storto, M., Gaviraghi, G., Terstappen, G. C. and Nicoletti, F. (2004). Induction of Dickkopf-1, a negative modulator of the Wnt pathway, is associated with neuronal degeneration in Alzheimer's brain. *J Neurosci* 24, 6021-7.

Caspersen, C., Wang, N., Yao, J., Sosunov, A., Chen, X., Lustbader, J. W., Xu, H. W., Stern, D., McKhann, G. and Yan, S. D. (2005). Mitochondrial Abeta: a potential focal point for neuronal metabolic dysfunction in Alzheimer's disease. *FASEB J* 19, 2040-1.

Cecconi, F., Di Bartolomeo, S., Nardacci, R., Fuoco, C., Corazzari, M., Giunta, L., Romagnoli, A., Stoykova, A., Chowdhury, K., Fimia, G. M. et al. (2007). A novel role for autophagy in neurodevelopment. *Autophagy* 3, 506-8.

Chang, L. and Karin, M. (2001). Mammalian MAP kinase signalling cascades. *Nature* 410, 37-40.

Chang, M. Y., Sun, W., Ochiai, W., Nakashima, K., Kim, S. Y., Park, C. H., Kang, J. S., Shim, J. W., Jo, A. Y., Kang, C. S. et al. (2007). Bcl-XL/Bax proteins direct the fate of embryonic cortical precursor cells. *Mol Cell Biol* 27, 4293-305.

Chasis, J. A. and Schrier, S. L. (1989). Membrane deformability and the capacity for shape change in the erythrocyte. *Blood* 74, 2562-8.

Chatterjee, A., Upadhyay, S., Chang, X., Nagpal, J. K., Trink, B. and Sidransky, D. (2008). U-box-type ubiquitin E4 ligase, UFD2a attenuates cisplatin mediated degradation of DeltaNp63alpha. *Cell cycle* 7, 1231-7.

Chen, N. and Karantza, V. (2011). Autophagy as a therapeutic target in cancer. *Cancer Biol Ther* 11, 157-68.

Chen, X., Vega, V. B. and Ng, H. H. (2008). Transcriptional regulatory networks in embryonic stem cells. *Cold Spring Harb Symp Quant Biol* 73, 203-9.

Chen, Y., Azad, M. B. and Gibson, S. B. (2009). Superoxide is the major reactive oxygen species regulating autophagy. *Cell Death Differ* 16, 1040-52.

Chen, Y. and Dong, C. (2009). Abeta40 promotes neuronal cell fate in neural progenitor cells. *Cell Death Differ* 16, 386-94.

Cheung, Y. T., Zhang, N. Q., Hung, C. H., Lai, C. S., Yu, M. S., So, K. F. and Chang, R. C. (2011). Temporal relationship of autophagy and apoptosis in neurons challenged by low molecular weight β-amyloid peptide. *J Cell Mol Med* 15, 244-57.

Chevallier, N. L., Soriano, S., Kang, D. E., Masliah, E., Hu, G. and Koo, E. H. (2005). Perturbed neurogenesis in the adult hippocampus associated with presenilin-1 A246E mutation. *Am J Pathol* 167, 151-9.

Chinenov, Y. and Kerppola, T. K. (2001). Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene* 20, 2438-52.

Chipuk, J. E., Bouchier-Hayes, L., Kuwana, T., Newmeyer, D. D. and Green, D. R. (2005). PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. *Science* 309, 1732-5.

Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M. and Green, D. R. (2004). Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* 303, 1010-4.

Choi, B. H. and Lapham, L. W. (1978). Radial glia in the human fetal cerebrum: a combined Golgi, immunofluorescent and electron microscopic study. *Brain Res* 148, 295-311.

Christie, B. R. and Cameron, H. A. (2006). Neurogenesis in the adult hippocampus. *Hippocampus* 16, 199-207.

Collavin, L., Lunardi, A. and Del Sal, G. (2010). p53-family proteins and their regulators: hubs and spokes in tumor suppression. *Cell Death Differ* 17, 901-11.

Conti, L. and Cattaneo, E. (2010). Neural stem cell systems: physiological players or in vitro entities? *Nat Rev Neurosci* 11, 176-87.

Conti, L., Pollard, S. M., Gorba, T., Reitano, E., Toselli, M., Biella, G., Sun, Y., Sanzone, S., Ying, Q.-L., Cattaneo, E. et al. (2005). Niche-Independent Symmetrical Self-Renewal of a Mammalian Tissue Stem Cell. *PLoS Biol* 3, e283.

Courtois, S., de Fromentel, C. C. and Hainaut, P. (2004). p53 protein variants: structural and functional similarities with p63 and p73 isoforms. *Oncogene* 23, 631-8.

Cregan, S. P., Arbour, N. A., Maclaurin, J. G., Callaghan, S. M., Fortin, A., Cheung, E. C., Guberman, D. S., Park, D. S. and Slack, R. S. (2004). p53 activation domain 1 is essential for PUMA upregulation and p53-mediated neuronal cell death. *J Neurosci* 24, 10003-12.

Crighton, D., Wilkinson, S., O'Prey, J., Syed, N., Smith, P., Harrison, P. R., Gasco, M., Garrone, O., Crook, T. and Ryan, K. M. (2006). DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. *Cell* 126, 121-34.

Culmsee, C., Zhu, X., Yu, Q. S., Chan, S. L., Camandola, S., Guo, Z., Greig, N. H. and Mattson, M. P. (2001). A synthetic inhibitor of p53 protects neurons against death induced by ischemic and excitotoxic insults, and amyloid beta-peptide. *J Neurochem* 77, 220-8.

Danial, N. N. and Korsmeyer, S. J. (2004). Cell death: critical control points. *Cell* 116, 205-19.

Dawson, G. R., Seabrook, G. R., Zheng, H., Smith, D. W., Graham, S., O'Dowd, G., Bowery, B. J., Boyce, S., Trumbauer, M. E., Chen, H. Y. et al. (1999). Age-related

cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the beta-amyloid precursor protein. *Neuroscience* 90, 1-13.

De Laurenzi, V., Raschellá, G., Barcaroli, D., Annicchiarico-Petruzzelli, M., Ranalli, M., Catani, M. V., Tanno, B., Costanzo, A., Levrero, M. and Melino, G. (2000). Induction of neuronal differentiation by p73 in a neuroblastoma cell line. *J Biol Chem* 275, 15226-31.

Debatin, K. M. and Krammer, P. H. (2004). Death receptors in chemotherapy and cancer. *Oncogene* 23, 2950-66.

Dechant, G. and Barde, Y. A. (2002). The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. *Nat Neurosci* 5, 1131-6.

Di Giovanni, S., Knights, C. D., Rao, M., Yakovlev, A., Beers, J., Catania, J., Avantaggiati, M. L. and Faden, A. I. (2006). The tumor suppressor protein p53 is required for neurite outgrowth and axon regeneration. *EMBO J* 25, 4084-96.

Dietz, S., Rother, K., Bamberger, C., Schmale, H., Mössner, J. and Engeland, K. (2002). Differential regulation of transcription and induction of programmed cell death by human p53-family members p63 and p73. *FEBS Lett* 525, 93-9.

Doetsch, F. (2003). A niche for adult neural stem cells. *Curr Opin Genet Dev* 13, 543-50.

Doetsch, F., Caillé, I., Lim, D. A., García-Verdugo, J. M. and Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97, 703-16.

Duan, W. M., Rodrigues, C. M., Zhao, L. R., Steer, C. J., Low, W. C. and Rodrigures, C. M. (2002). Tauroursodeoxycholic acid improves the survival and function of nigral transplants in a rat model of Parkinson's disease. *Cell Transplant* 11, 195-205.

Dugani, C. B., Paquin, A., Fujitani, M., Kaplan, D. R. and Miller, F. D. (2009). p63 antagonizes p53 to promote the survival of embryonic neural precursor cells. *J Neurosci* 29, 6710-21.

Dulloo, I., Gopalan, G., Melino, G. and Sabapathy, K. (2010). The antiapoptotic DeltaNp73 is degraded in a c-Jun-dependent manner upon genotoxic stress through the antizyme-mediated pathway. *Proc Natl Acad Sci U S A* 107, 4902-7.

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

Dötsch, V., Bernassola, F., Coutandin, D., Candi, E. and Melino, G. (2010). p63 and p73, the ancestors of p53. *Cold Spring Harb Perspect Biol* 2, a004887.

Edinger, A. L. and Thompson, C. B. (2003). Defective autophagy leads to cancer. *Cancer Cell* 4, 422-4.

Edwards, S. N. and Tolkovsky, A. M. (1994). Characterization of apoptosis in cultured rat sympathetic neurons after nerve growth factor withdrawal. *J Cell Biol* 124, 537-46.

Eferl, R. and Wagner, E. F. (2003). AP-1: a double-edged sword in tumorigenesis. *Nat Rev Cancer* 3, 859-68.

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

Eilers, A., Whitfield, J., Shah, B., Spadoni, C., Desmond, H. and Ham, J. (2001). Direct inhibition of c-Jun N-terminal kinase in sympathetic neurones prevents c-jun promoter activation and NGF withdrawal-induced death. *J Neurochem* 76, 1439-54.

Eisenberg-Lerner, A., Bialik, S., Simon, H. U. and Kimchi, A. (2009). Life and death partners: apoptosis, autophagy and the cross-talk between them. *Cell Death Differ* 16, 966-75.

Elder, G. A., Gama Sosa, M. A. and De Gasperi, R. (2010). Transgenic mouse models of Alzheimer's disease. *Mt Sinai J Med* 77, 69-81.

Eom, D. S., Choi, W. S. and Oh, Y. J. (2004). Bcl-2 enhances neurite extension via activation of c-Jun N-terminal kinase. *Biochem Biophys Res Commun* 314, 377-81.

Eriksson, P. S., Perfilieva, E., Björk-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A. and Gage, F. H. (1998). Neurogenesis in the adult human hippocampus. *Nat Med* 4, 1313-7.

Essick, E. E. and Sam, F. (2010). Oxidative stress and autophagy in cardiac disease, neurological disorders, aging and cancer. *Oxid Med Cell Longev* 3, 168-77.

Estus, S., Tucker, H. M., van Rooyen, C., Wright, S., Brigham, E. F., Wogulis, M. and Rydel, R. E. (1997). Aggregated amyloid-beta protein induces cortical neuronal apoptosis and concomitant "apoptotic" pattern of gene induction. *J Neurosci* 17, 7736-45.

Estus, S., Zaks, W. J., Freeman, R. S., Gruda, M., Bravo, R. and Johnson, E. M. (1994). Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. *J Cell Biol* 127, 1717-27.

Evans, M. J. and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154-6.

Ezashi, T., Das, P. and Roberts, R. M. (2005). Low O2 tensions and the prevention of differentiation of hES cells. *Proc Natl Acad Sci U S A* 102, 4783-8.

Feng, L., Hatten, M. E. and Heintz, N. (1994). Brain lipid-binding protein (BLBP): a novel signaling system in the developing mammalian CNS. *Neuron* 12, 895-908.

Fernando, P., Brunette, S. and Megeney, L. A. (2005). Neural stem cell differentiation is dependent upon endogenous caspase 3 activity. *Faseb J* 19, 1671-3.

Fernando, P., Kelly, J. F., Balazsi, K., Slack, R. S. and Megeney, L. A. (2002). Caspase 3 activity is required for skeletal muscle differentiation. *Proc Natl Acad Sci U S A* 99, 11025-30.

Fernando, P. and Megeney, L. A. (2007). Is caspase-dependent apoptosis only cell differentiation taken to the extreme? *FASEB J* 21, 8-17.

Ferreira, A. and Kosik, K. S. (1996). Accelerated neuronal differentiation induced by p53 suppression. *J Cell Sci* 109 (Pt 6), 1509-16.

Fimia, G. M., Stoykova, A., Romagnoli, A., Giunta, L., Di Bartolomeo, S., Nardacci, R., Corazzari, M., Fuoco, C., Ucar, A., Schwartz, P. et al. (2007). Ambra1 regulates autophagy and development of the nervous system. *Nature* 447, 1121-5.

Flores, E. R., Tsai, K. Y., Crowley, D., Sengupta, S., Yang, A., McKeon, F. and Jacks, T. (2002). p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. *Nature* 416, 560-4.

Fonseca, M. B., Nunes, A. F. and Rodrigues, C. M. (2012). c-Jun Regulates the Stability of Anti-Apoptotic ΔNp63 in Amyloid-β-Induced Apoptosis. *J Alzheimers Dis* 28, 685-94.

Fuchs, S. Y., Adler, V., Pincus, M. R. and Ronai, Z. (1998). MEKK1/JNK signaling stabilizes and activates p53. *Proc Natl Acad Sci U S A* 95, 10541-6.

Fujitani, M., Cancino, G. I., Dugani, C. B., Weaver, I. C., Gauthier-Fisher, A., Paquin, A., Mak, T. W., Wojtowicz, M. J., Miller, F. D. and Kaplan, D. R. (2010). TAp73 acts via the bHLH Hey2 to promote long-term maintenance of neural precursors. *Curr Biol* 20, 2058-65.

Fulda, S. and Vucic, D. (2012). Targeting IAP proteins for therapeutic intervention in cancer. *Nat Rev Drug Discov* 11, 109-24.

Gage, F. H. (2000). Mammalian neural stem cells. *Science* 287, 1433-8.

Galli, F., Rossi, M., D'Alessandra, Y., De Simone, M., Lopardo, T., Haupt, Y., Alsheich-Bartok, O., Anzi, S., Shaulian, E., Calabro, V. et al. (2010). MDM2 and Fbw7 cooperate to induce p63 protein degradation following DNA damage and cell differentiation. *Journal of cell science* 123, 2423-33.

Galluzzi, L., Kepp, O., Trojel-Hansen, C. and Kroemer, G. (2012). Non-apoptotic functions of apoptosis-regulatory proteins. *EMBO Rep* 13, 322-30.

Galluzzi, L., Morselli, E., Kepp, O., Vitale, I., Pinti, M. and Kroemer, G. (2011). Mitochondrial liaisons of p53. *Antioxid Redox Signal* 15, 1691-714.

Gandy, S. (2005). The role of cerebral amyloid beta accumulation in common forms of Alzheimer disease. *J Clin Invest* 115, 1121-9.

Geisler, S., Holmström, K. M., Skujat, D., Fiesel, F. C., Rothfuss, O. C., Kahle, P. J. and Springer, W. (2010). PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol* 12, 119-31.

Gil-Perotin, S., Marin-Husstege, M., Li, J., Soriano-Navarro, M., Zindy, F., Roussel, M. F., Garcia-Verdugo, J. M. and Casaccia-Bonnefil, P. (2006). Loss of p53 induces changes in the behavior of subventricular zone cells: implication for the genesis of glial tumors. *J Neurosci* 26, 1107-16.

Giuffrida, M. L., Caraci, F., Pignataro, B., Cataldo, S., De Bona, P., Bruno, V., Molinaro, G., Pappalardo, G., Messina, A., Palmigiano, A. et al. (2009). Beta-amyloid monomers are neuroprotective. *J Neurosci* 29, 10582-7.

Glaser, T., Pollard, S. M., Smith, A. and Brustle, O. (2007). Tripotential differentiation of adherently expandable neural stem (NS) cells. *PLoS One* 2, e298.

Gong, J. G., Costanzo, A., Yang, H. Q., Melino, G., Kaelin, W. G., Levrero, M. and Wang, J. Y. (1999). The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. *Nature* 399, 806-9.

Gonzalez-Cano, L., Herreros-Villanueva, M., Fernandez-Alonso, R., Ayuso-Sacido, A., Meyer, G., Garcia-Verdugo, J. M., Silva, A., Marques, M. M. and Marin, M. C. (2010). p73 deficiency results in impaired self renewal and premature neuronal differentiation of mouse neural progenitors independently of p53. *Cell Death Dis* 1, e109.

Gozuacik, D. and Kimchi, A. (2004). Autophagy as a cell death and tumor suppressor mechanism. *Oncogene* 23, 2891-906.

Gregori, L., Fuchs, C., Figueiredo-Pereira, M. E., Van Nostrand, W. E. and Goldgaber, D. (1995). Amyloid beta-protein inhibits ubiquitin-dependent protein degradation in vitro. *J Biol Chem* 270, 19702-8.

Gressner, O., Schilling, T., Lorenz, K., Schulze Schleithoff, E., Koch, A., Schulze-Bergkamen, H., Lena, A. M., Candi, E., Terrinoni, A., Catani, M. V. et al. (2005).

TAp63alpha induces apoptosis by activating signaling via death receptors and mitochondria. *EMBO J* 24, 2458-71.

Gurusamy, N. and Das, D. K. (2009). Autophagy, redox signaling, and ventricular remodeling. *Antioxid Redox Signal* 11, 1975-88.

Götz, M. and Huttner, W. B. (2005). The cell biology of neurogenesis. *Nat Rev Mol Cell Biol* 6, 777-88.

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

Han, M. K., Song, E. K., Guo, Y., Ou, X., Mantel, C. and Broxmeyer, H. E. (2008). SIRT1 regulates apoptosis and Nanog expression in mouse embryonic stem cells by controlling p53 subcellular localization. *Cell Stem Cell* 2, 241-51.

Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H. et al. (2006). Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* 441, 885-9.

Hardy, J. and Selkoe, D. J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297, 353-6.

Harris, C. A. and Johnson, E. M. (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, 37754-60.

Harris, S. L. and Levine, A. J. (2005). The p53 pathway: positive and negative feedback loops. *Oncogene* 24, 2899-908.

Haubensak, W., Attardo, A., Denk, W. and Huttner, W. B. (2004). Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. *Proc Natl Acad Sci U S A* 101, 3196-201.

Haughey, N. J., Liu, D., Nath, A., Borchard, A. C. and Mattson, M. P. (2002a). Disruption of neurogenesis in the subventricular zone of adult mice, and in human cortical neuronal precursor cells in culture, by amyloid beta-peptide: implications for the pathogenesis of Alzheimer's disease. *Neuromolecular Med* 1, 125-35.

Haughey, N. J., Nath, A., Chan, S. L., Borchard, A. C., Rao, M. S. and Mattson, M. P. (2002b). Disruption of neurogenesis by amyloid beta-peptide, and perturbed neural progenitor cell homeostasis, in models of Alzheimer's disease. *J Neurochem* 83, 1509-24.

He, Y., Cui, J., Lee, J. C., Ding, S., Chalimoniuk, M., Simonyi, A., Sun, A. Y., Gu, Z., Weisman, G. A., Wood, W. G. et al. (2011). Prolonged exposure of cortical neurons to oligomeric amyloid-β impairs NMDA receptor function via NADPH oxidase-mediated ROS production: protective effect of green tea (-)-epigallocatechin-3-gallate. *ASN Neuro* 3, e00050.

Helton, E. S., Zhu, J. and Chen, X. (2006). The unique NH2-terminally deleted (DeltaN) residues, the PXXP motif, and the PPXY motif are required for the transcriptional activity of the DeltaN variant of p63. *J Biol Chem* 281, 2533-42.

Heo, C., Chang, K. A., Choi, H. S., Kim, H. S., Kim, S., Liew, H., Kim, J. A., Yu, E., Ma, J. and Suh, Y. H. (2007). Effects of the monomeric, oligomeric, and fibrillar Abeta42 peptides on the proliferation and differentiation of adult neural stem cells from subventricular zone. *J Neurochem* 102, 493-500.

Herdegen, T., Skene, P. and Bähr, M. (1997). The c-Jun transcription factor--bipotential mediator of neuronal death, survival and regeneration. *Trends Neurosci* 20, 227-31.

Hernández-Acosta, N. C., Cabrera-Socorro, A., Morlans, M. P., Delgado, F. J., Suárez-Solá, M. L., Sottocornola, R., Lu, X., González-Gómez, M. and Meyer, G. (2011). Dynamic expression of the p53 family members p63 and p73 in the mouse and human telencephalon during development and in adulthood. *Brain Res* 1372, 29-40.

Hershkovitz Rokah, O., Shpilberg, O. and Granot, G. (2010). NAD(P)H quinone oxidoreductase protects TAp63gamma from proteasomal degradation and regulates TAp63gamma-dependent growth arrest. *PloS one* 5, e11401.

Hess, J., Angel, P. and Schorpp-Kistner, M. (2004). AP-1 subunits: quarrel and harmony among siblings. *Journal of cell science* 117, 5965-73.

Hilberg, F., Aguzzi, A., Howells, N. and Wagner, E. F. (1993). c-jun is essential for normal mouse development and hepatogenesis. *Nature* 365, 179-81.

Holembowski, L., Schulz, R., Talos, F., Scheel, A., Wolff, S., Dobbelstein, M. and Moll, U. (2011). While p73 is essential, p63 is completely dispensable for the development of the central nervous system. *Cell Cycle* 10, 680-9.

Hooper, C., Meimaridou, E., Tavassoli, M., Melino, G., Lovestone, S. and Killick, R. (2007). p53 is upregulated in Alzheimer's disease and induces tau phosphorylation in HEK293a cells. *Neurosci Lett* 418, 34-7.

Huang, J., Sengupta, R., Espejo, A. B., Lee, M. G., Dorsey, J. A., Richter, M., Opravil, S., Shiekhattar, R., Bedford, M. T., Jenuwein, T. et al. (2007). p53 is regulated by the lysine demethylase LSD1. *Nature* 449, 105-8.

Huang, W. P. and Klionsky, D. J. (2002). Autophagy in yeast: a review of the molecular machinery. *Cell Struct Funct* 27, 409-20.

Hudson, C. D., Morris, P. J., Latchman, D. S. and Budhram-Mahadeo, V. S. (2005). Brn-3a transcription factor blocks p53-mediated activation of proapoptotic target genes Noxa and Bax in vitro and in vivo to determine cell fate. *J Biol Chem* 280, 11851-8.

Hudson, C. D., Podesta, J., Henderson, D., Latchman, D. S. and Budhram-Mahadeo, V. (2004). Coexpression of Brn-3a POU protein with p53 in a population of neuronal progenitor cells is associated with differentiation and protection against apoptosis. *J Neurosci Res* 78, 803-14.

Hughes, A. L., Gollapudi, L., Sladek, T. L. and Neet, K. E. (2000). Mediation of nerve growth factor-driven cell cycle arrest in PC12 cells by p53. Simultaneous differentiation and proliferation subsequent to p53 functional inactivation. *J Biol Chem* 275, 37829-37.

Hung, S. Y., Huang, W. P., Liou, H. C. and Fu, W. M. (2009). Autophagy protects neuron from Abeta-induced cytotoxicity. *Autophagy* 5, 502-10.

Ip, Y. T. and Davis, R. J. (1998). Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. *Curr Opin Cell Biol* 10, 205-19.

Irwin, M. S. and Kaelin, W. G. (2001). p53 family update: p73 and p63 develop their own identities. *Cell Growth Differ* 12, 337-49.

Itagaki, S., McGeer, P. L., Akiyama, H., Zhu, S. and Selkoe, D. (1989). Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease. *J Neuroimmunol* 24, 173-82.

Iwakuma, T. and Lozano, G. (2003). MDM2, an introduction. *Mol Cancer Res* 1, 993-1000.

Iwasaki, K., Sunderland, T., Kusiak, J. W. and Wolozin, B. (1996). Changes in gene transcription during a beta-mediated cell death. *Mol Psychiatry* 1, 65-71.

Iwata, A., Christianson, J. C., Bucci, M., Ellerby, L. M., Nukina, N., Forno, L. S. and Kopito, R. R. (2005). Increased susceptibility of cytoplasmic over nuclear polyglutamine aggregates to autophagic degradation. *Proc Natl Acad Sci U S A* 102, 13135-40.

Jacobs, W. B., Govoni, G., Ho, D., Atwal, J. K., Barnabe-Heider, F., Keyes, W. M., Mills, A. A., Miller, F. D. and Kaplan, D. R. (2005). p63 is an essential proapoptotic protein during neural development. *Neuron* 48, 743-56.

Jacobs, W. B., Kaplan, D. R. and Miller, F. D. (2006). The p53 family in nervous system development and disease. *J Neurochem* 97, 1571-84.

Jiang, P., Du, W., Heese, K. and Wu, M. (2006). The Bad guy cooperates with good cop p53: Bad is transcriptionally up-regulated by p53 and forms a Bad/p53 complex at the mitochondria to induce apoptosis. *Mol Cell Biol* 26, 9071-82.

Jin, K., Galvan, V., Xie, L., Mao, X. O., Gorostiza, O. F., Bredesen, D. E. and Greenberg, D. A. (2004). Enhanced neurogenesis in Alzheimer's disease transgenic (PDGF-APPSw,Ind) mice. *Proc Natl Acad Sci U S A* 101, 13363-7.

Jones, E. V., Dickman, M. J. and Whitmarsh, A. J. (2007). Regulation of p73-mediated apoptosis by c-Jun N-terminal kinase. *Biochem J* 405, 617-23.

Jost, C. A., Marin, M. C. and Kaelin, W. G. (1997). p73 is a simian [correction of human] p53-related protein that can induce apoptosis. *Nature* 389, 191-4.

Junyent, F., de Lemos, L., Verdaguer, E., Folch, J., Ferrer, I., Ortuño-Sahagún, D., Beas-Zárate, C., Romero, R., Pallàs, M., Auladell, C. et al. (2011). Gene expression profile in JNK3 null mice: a novel specific activation of the PI3K/AKT pathway. *J Neurochem* 117, 244-52.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y. and Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 19, 5720-8.

Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K., Ohsumi, M. and Ohsumi, Y. (2000). Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J Cell Biol* 150, 1507-13.

Kang, R., Zeh, H. J., Lotze, M. T. and Tang, D. (2011). The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ* 18, 571-80.

Kaplan, D. R. and Miller, F. D. (2000). Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol* 10, 381-91.

Kaplan, M. S. and Bell, D. H. (1984). Mitotic neuroblasts in the 9-day-old and 11-month-old rodent hippocampus. *J Neurosci* 4, 1429-41.

Karin, M., Liu, Z. and Zandi, E. (1997). AP-1 function and regulation. *Curr Opin Cell Biol* 9, 240-6.

Keene, C. D., Rodrigues, C. M., Eich, T., Chhabra, M. S., Steer, C. J. and Low, W. C. (2002). Tauroursodeoxycholic acid, a bile acid, is neuroprotective in a transgenic animal model of Huntington's disease. *Proc Natl Acad Sci U S A* 99, 10671-6.

Kelly, P. N. and Strasser, A. (2011). The role of Bcl-2 and its pro-survival relatives in tumourigenesis and cancer therapy. *Cell Death Differ* 18, 1414-24.

Kempermann, G. and Gage, F. H. (2000). Neurogenesis in the adult hippocampus. *Novartis Found Symp* 231, 220-35; discussion 235-41, 302-6.

Kerr, J. F., Wyllie, A. H. and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26, 239-57.

Khandelwal, P. J., Herman, A. M., Hoe, H. S., Rebeck, G. W. and Moussa, C. E. (2011). Parkin mediates beclin-dependent autophagic clearance of defective mitochondria and ubiquitinated Abeta in AD models. *Hum Mol Genet* 20, 2091-102.

Kihiko, M. E., Tucker, H. M., Rydel, R. E. and Estus, S. (1999). c-Jun contributes to amyloid beta-induced neuronal apoptosis but is not necessary for amyloid beta-induced c-jun induction. *J Neurochem* 73, 2609-12.

Kim, J., Onstead, L., Randle, S., Price, R., Smithson, L., Zwizinski, C., Dickson, D. W., Golde, T. and McGowan, E. (2007). Abeta40 inhibits amyloid deposition in vivo. *J Neurosci* 27, 627-33.

Kirschner, R. D., Sanger, K., Muller, G. A. and Engeland, K. (2008). Transcriptional activation of the tumor suppressor and differentiation gene S100A2 by a novel p63-binding site. *Nucleic acids research* 36, 2969-80.

Kitazawa, M., Oddo, S., Yamasaki, T. R., Green, K. N. and LaFerla, F. M. (2005). Lipopolysaccharide-induced inflammation exacerbates tau pathology by a cyclin-dependent kinase 5-mediated pathway in a transgenic model of Alzheimer's disease. *J Neurosci* 25, 8843-53.

Klein, W. L. (2002). Abeta toxicity in Alzheimer's disease: globular oligomers (ADDLs) as new vaccine and drug targets. *Neurochem Int* 41, 345-52.

Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., Ueno, T., Koike, M., Uchiyama, Y., Kominami, E. et al. (2006). Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 441, 880-4.

Kontush, A., Donarski, N. and Beisiegel, U. (2001). Resistance of human cerebrospinal fluid to in vitro oxidation is directly related to its amyloid-beta content. *Free Radic Res* 35, 507-17.

Kornack, D. R. and Rakic, P. (2001). The generation, migration, and differentiation of olfactory neurons in the adult primate brain. *Proc Natl Acad Sci U S A* 98, 4752-7.

Krizhanovsky, V. and Lowe, S. W. (2009). Stem cells: The promises and perils of p53. *Nature* 460, 1085-6.

Kroemer, G., Galluzzi, L. and Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. *Physiol Rev* 87, 99-163.

Krstic, D. and Knuesel, I. (2013). Deciphering the mechanism underlying late-onset Alzheimer disease. *Nat Rev Neurol* 9, 25-34.

Kruse, J. P. and Gu, W. (2009). Modes of p53 regulation. *Cell* 137, 609-22.

Kuan, C. Y., Yang, D. D., Samanta Roy, D. R., Davis, R. J., Rakic, P. and Flavell, R. A. (1999). The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron* 22, 667-76.

Kuhn, H. G., Dickinson-Anson, H. and Gage, F. H. (1996). Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J Neurosci* 16, 2027-33.

Kuida, K., Haydar, T. F., Kuan, C. Y., Gu, Y., Taya, C., Karasuyama, H., Su, M. S., Rakic, P. and Flavell, R. A. (1998). Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* 94, 325-37.

Kuida, K., Zheng, T. S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P. and Flavell, R. A. (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* 384, 368-72.

Kukekov, V. G., Laywell, E. D., Suslov, O., Davies, K., Scheffler, B., Thomas, L. B., O'Brien, T. F., Kusakabe, M. and Steindler, D. A. (1999). Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain. *Exp Neurol* 156, 333-44.

Kumar-Singh, S., Theuns, J., Van Broeck, B., Pirici, D., Vennekens, K., Corsmit, E., Cruts, M., Dermaut, B., Wang, R. and Van Broeckhoven, C. (2006). Mean age-of-onset of familial alzheimer disease caused by presenilin mutations correlates with both increased Abeta42 and decreased Abeta40. *Hum Mutat* 27, 686-95.

Kuwabara, T., Hsieh, J., Nakashima, K., Taira, K. and Gage, F. H. (2004). A small modulatory dsRNA specifies the fate of adult neural stem cells. *Cell* 116, 779-93.

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

LaFerla, F. M. (2010). Pathways linking Abeta and tau pathologies. *Biochem Soc Trans* 38, 993-5.

LaFerla, F. M., Hall, C. K., Ngo, L. and Jay, G. (1996). Extracellular deposition of beta-amyloid upon p53-dependent neuronal cell death in transgenic mice. *J Clin Invest* 98, 1626-32.

Laird, F. M., Cai, H., Savonenko, A. V., Farah, M. H., He, K., Melnikova, T., Wen, H., Chiang, H. C., Xu, G., Koliatsos, V. E. et al. (2005). BACE1, a major determinant of selective vulnerability of the brain to amyloid-beta amyloidogenesis, is essential for cognitive, emotional, and synaptic functions. *J Neurosci* 25, 11693-709.

Lanni, C., Nardinocchi, L., Puca, R., Stanga, S., Uberti, D., Memo, M., Govoni, S., D'Orazi, G. and Racchi, M. (2010). Homeodomain interacting protein kinase 2: a target for Alzheimer's beta amyloid leading to misfolded p53 and inappropriate cell survival. *PLoS One* 5, e10171.

Lassmann, H., Bancher, C., Breitschopf, H., Wegiel, J., Bobinski, M., Jellinger, K. and Wisniewski, H. M. (1995). Cell death in Alzheimer's disease evaluated by DNA fragmentation in situ. *Acta Neuropathol* 89, 35-41.

Laurén, J., Gimbel, D. A., Nygaard, H. B., Gilbert, J. W. and Strittmatter, S. M. (2009). Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. *Nature* 457, 1128-32.

Lavin, M. F. and Gueven, N. (2006). The complexity of p53 stabilization and activation. *Cell Death Differ* 13, 941-50.

Lazaridis, K. N., Gores, G. J. and Lindor, K. D. (2001). Ursodeoxycholic acid 'mechanisms of action and clinical use in hepatobiliary disorders'. *J Hepatol* 35, 134-46.

Lazzari, C., Prodosmo, A., Siepi, F., Rinaldo, C., Galli, F., Gentileschi, M., Bartolazzi, A., Costanzo, A., Sacchi, A., Guerrini, L. et al. (2011). HIPK2 phosphorylates DeltaNp63alpha and promotes its degradation in response to DNA damage. *Oncogene*.

Lee, A. F., Ho, D. K., Zanassi, P., Walsh, G. S., Kaplan, D. R. and Miller, F. D. (2004). Evidence that DeltaNp73 promotes neuronal survival by p53-dependent and p53-independent mechanisms. *J Neurosci* 24, 9174-84.

Levine, B. and Klionsky, D. J. (2004). Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell* 6, 463-77.

Levine, B. and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. *Cell* 132, 27-42.

Levine, B. and Ranganathan, R. (2010). Autophagy: Snapshot of the network. *Nature* 466, 38-40.

Levitt, P. and Rakic, P. (1980). Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain. *J Comp Neurol* 193, 815-40.

Li, D. D., Wang, L. L., Deng, R., Tang, J., Shen, Y., Guo, J. F., Wang, Y., Xia, L. P., Feng, G. K., Liu, Q. Q. et al. (2009). The pivotal role of c-Jun NH2-terminal kinase-mediated Beclin 1 expression during anticancer agents-induced autophagy in cancer cells. *Oncogene* 28, 886-98.

Li, X. and Zhao, X. (2008). Epigenetic regulation of mammalian stem cells. *Stem Cells Dev* 17, 1043-52.

Liefer, K. M., Koster, M. I., Wang, X. J., Yang, A., McKeon, F. and Roop, D. R. (2000). Down-regulation of p63 is required for epidermal UV-B-induced apoptosis. *Cancer research* 60, 4016-20.

Lin, K. W., Nam, S. Y., Toh, W. H., Dulloo, I. and Sabapathy, K. (2004). Multiple stress signals induce p73beta accumulation. *Neoplasia* 6, 546-57.

Lin, T., Chao, C., Saito, S., Mazur, S. J., Murphy, M. E., Appella, E. and Xu, Y. (2005). p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat Cell Biol* 7, 165-71.

Ling, Y., Morgan, K. and Kalsheker, N. (2003). Amyloid precursor protein (APP) and the biology of proteolytic processing: relevance to Alzheimer's disease. *Int J Biochem Cell Biol* 35, 1505-35.

Lipinski, M. M., Zheng, B., Lu, T., Yan, Z., Py, B. F., Ng, A., Xavier, R. J., Li, C., Yankner, B. A., Scherzer, C. R. et al. (2010). Genome-wide analysis reveals mechanisms modulating autophagy in normal brain aging and in Alzheimer's disease. *Proc Natl Acad Sci U S A* 107, 14164-9.

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, 28873-7.

Lois, C. and Alvarez-Buylla, A. (1993). Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc Natl Acad Sci U S A* 90, 2074-7.

Lois, C. and Alvarez-Buylla, A. (1994). Long-distance neuronal migration in the adult mammalian brain. *Science* 264, 1145-8.

Lois, C., García-Verdugo, J. M. and Alvarez-Buylla, A. (1996). Chain migration of neuronal precursors. *Science* 271, 978-81.

Longatti, A. and Tooze, S. A. (2009). Vesicular trafficking and autophagosome formation. *Cell Death Differ* 16, 956-65.

Loo, D. T., Copani, A., Pike, C. J., Whittemore, E. R., Walencewicz, A. J. and Cotman, C. W. (1993). Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. *Proceedings of the National Academy of Sciences of the United States of America* 90, 7951-5.

Lopez-Toledano, M. A. and Shelanski, M. L. (2004). Neurogenic effect of beta-amyloid peptide in the development of neural stem cells. *J Neurosci* 24, 5439-44.

Lorent, K., Overbergh, L., Moechars, D., De Strooper, B., Van Leuven, F. and Van den Berghe, H. (1995). Expression in mouse embryos and in adult mouse brain of three members of the amyloid precursor protein family, of the alpha-2-macroglobulin receptor/low density lipoprotein receptor-related protein and of its ligands apolipoprotein E, lipoprotein lipase, alpha-2-macroglobulin and the 40,000 molecular weight receptor-associated protein. *Neuroscience* 65, 1009-25.

Lu, C., Fu, W., Salvesen, G. S. and Mattson, M. P. (2002). Direct cleavage of AMPA receptor subunit GluR1 and suppression of AMPA currents by caspase-3: implications for synaptic plasticity and excitotoxic neuronal death. *Neuromolecular Med* 1, 69-79.

Lum, J. J., Bauer, D. E., Kong, M., Harris, M. H., Li, C., Lindsten, T. and Thompson, C. B. (2005). Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell* 120, 237-48.

Luo, Y., Sunderland, T. and Wolozin, B. (1996). Physiologic levels of beta-amyloid activate phosphatidylinositol 3-kinase with the involvement of tyrosine phosphorylation. *J Neurochem* 67, 978-87.

Luo, Y., Umegaki, H., Wang, X., Abe, R. and Roth, G. S. (1998). Dopamine induces apoptosis through an oxidation-involved SAPK/JNK activation pathway. *J Biol Chem* 273, 3756-64.

Luskin, M. B. (1993). Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron* 11, 173-89.

López-Toledano, M. A. and Shelanski, M. L. (2004). Neurogenic effect of beta-amyloid peptide in the development of neural stem cells. *J Neurosci* 24, 5439-44.

Maiuri, M. C., Zalckvar, E., Kimchi, A. and Kroemer, G. (2007). Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol* 8, 741-52.

Malatesta, P., Appolloni, I. and Calzolari, F. (2008). Radial glia and neural stem cells. *Cell Tissue Res* 331, 165-78.

Malatesta, P., Hack, M. A., Hartfuss, E., Kettenmann, H., Klinkert, W., Kirchhoff, F. and Götz, M. (2003). Neuronal or glial progeny: regional differences in radial glia fate. *Neuron* 37, 751-64.

Mann, S. S. and Hammarback, J. A. (1994). Molecular characterization of light chain 3. A microtubule binding subunit of MAP1A and MAP1B. *J Biol Chem* 269, 11492-7.

Manning, A. M. and Davis, R. J. (2003). Targeting JNK for therapeutic benefit: from junk to gold? *Nat Rev Drug Discov* 2, 554-65.

Marcel, V., Petit, I., Murray-Zmijewski, F., Goullet de Rugy, T., Fernandes, K., Meuray, V., Diot, A., Lane, D. P., Aberdam, D. and Bourdon, J. C. (2012). Diverse p63 and p73 isoforms regulate Δ133p53 expression through modulation of the internal TP53 promoter activity. *Cell Death Differ* 19, 816-26.

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* 78, 7634-8.

Matrone, C., Di Luzio, A., Meli, G., D'Aguanno, S., Severini, C., Ciotti, M. T., Cattaneo, A. and Calissano, P. (2008). Activation of the amyloidogenic route by NGF deprivation induces apoptotic death in PC12 cells. *J Alzheimers Dis* 13, 81-96.

Mattson, M. P. (1997). Cellular actions of beta-amyloid precursor protein and its soluble and fibrillogenic derivatives. *Physiol Rev* 77, 1081-132.

Mattson, M. P. (2004). Pathways towards and away from Alzheimer's disease. *Nature* 430, 631-9.

Mattson, M. P. (2006). Neuronal life-and-death signaling, apoptosis, and neurodegenerative disorders. *Antioxid Redox Signal* 8, 1997-2006.

Mattson, M. P. and Goodman, Y. (1995). Different amyloidogenic peptides share a similar mechanism of neurotoxicity involving reactive oxygen species and calcium. *Brain Res* 676, 219-24.

Mazur-Kolecka, B., Golabek, A., Nowicki, K., Flory, M. and Frackowiak, J. (2006). Amyloid-beta impairs development of neuronal progenitor cells by oxidative mechanisms. *Neurobiol Aging* 27, 1181-92.

McGowan, E., Pickford, F., Kim, J., Onstead, L., Eriksen, J., Yu, C., Skipper, L., Murphy, M. P., Beard, J., Das, P. et al. (2005). Abeta42 is essential for parenchymal and vascular amyloid deposition in mice. *Neuron* 47, 191-9.

McKee, A. C., Kosik, K. S., Kennedy, M. B. and Kowall, N. W. (1990). Hippocampal neurons predisposed to neurofibrillary tangle formation are enriched in type II calcium/calmodulin-dependent protein kinase. *J Neuropathol Exp Neurol* 49, 49-63.

Mehta, P. D., Pirttila, T., Patrick, B. A., Barshatzky, M. and Mehta, S. P. (2001). Amyloid beta protein 1-40 and 1-42 levels in matched cerebrospinal fluid and plasma from patients with Alzheimer disease. *Neurosci Lett* 304, 102-6.

Meletis, K., Wirta, V., Hede, S. M., Nistér, M., Lundeberg, J. and Frisén, J. (2006). p53 suppresses the self-renewal of adult neural stem cells. *Development* 133, 363-9.

Melino, G., De Laurenzi, V. and Vousden, K. H. (2002). p73: Friend or foe in tumorigenesis. *Nat Rev Cancer* 2, 605-15.

Mellén, M. A., de la Rosa, E. J. and Boya, P. (2008). The autophagic machinery is necessary for removal of cell corpses from the developing retinal neuroepithelium. *Cell Death Differ* 15, 1279-90.

Merkle, F. T. and Alvarez-Buylla, A. (2006). Neural stem cells in mammalian development. *Curr Opin Cell Biol* 18, 704-9.

Merkle, F. T., Mirzadeh, Z. and Alvarez-Buylla, A. (2007). Mosaic organization of neural stem cells in the adult brain. *Science* 317, 381-4.

Merkle, F. T., Tramontin, A. D., García-Verdugo, J. M. and Alvarez-Buylla, A. (2004). Radial glia give rise to adult neural stem cells in the subventricular zone. *Proc Natl Acad Sci U S A* 101, 17528-32.

Merry, D. E., Veis, D. J., Hickey, W. F. and Korsmeyer, S. J. (1994). bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. *Development* 120, 301-11.

Michalak, E. M., Villunger, A., Adams, J. M. and Strasser, A. (2008). In several cell types tumour suppressor p53 induces apoptosis largely via Puma but Noxa can contribute. *Cell Death Differ* 15, 1019-29.

Millet, P., Lages, C. S., Haïk, S., Nowak, E., Allemand, I., Granotier, C. and Boussin, F. D. (2005). Amyloid-beta peptide triggers Fas-independent apoptosis and differentiation of neural progenitor cells. *Neurobiol Dis* 19, 57-65.

Mills, A. A. (2006). p63: oncogene or tumor suppressor? *Curr Opin Genet Dev* 16, 38-44.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M. and Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 113, 631-42.

Miyashita, T. and Reed, J. C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 80, 293-9.

Mizushima, N. (2005). A(beta) generation in autophagic vacuoles. *J Cell Biol* 171, 15-7.

Mizushima, N. and Levine, B. (2010). Autophagy in mammalian development and differentiation. *Nat Cell Biol* 12, 823-30.

Moll, U. M. and Slade, N. (2004). p63 and p73: roles in development and tumor formation. *Mol Cancer Res* 2, 371-86.

Moll, U. M., Wolff, S., Speidel, D. and Deppert, W. (2005). Transcription-independent pro-apoptotic functions of p53. *Curr Opin Cell Biol* 17, 631-6.

Moreira, P. I., Santos, R. X., Zhu, X., Lee, H. G., Smith, M. A., Casadesus, G. and Perry, G. (2010). Autophagy in Alzheimer's disease. *Expert Rev Neurother* 10, 1209-18.

Morselli, E., Galluzzi, L. and Kroemer, G. (2008). Mechanisms of p53-mediated mitochondrial membrane permeabilization. *Cell Res* 18, 708-10.

Mu, Y., Lee, S. W. and Gage, F. H. (2010). Signaling in adult neurogenesis. *Curr Opin Neurobiol* 20, 416-23.

Murray-Zmijewski, F., Lane, D. P. and Bourdon, J. C. (2006). p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. *Cell Death Differ* 13, 962-72.

Müller, M., Schleithoff, E. S., Stremmel, W., Melino, G., Krammer, P. H. and Schilling, T. (2006). One, two, three--p53, p63, p73 and chemosensitivity. *Drug Resist Updat* 9, 288-306.

Nedelsky, N. B., Todd, P. K. and Taylor, J. P. (2008). Autophagy and the ubiquitin-proteasome system: collaborators in neuroprotection. *Biochim Biophys Acta* 1782, 691-9.

Nicotera, P. and Melino, G. (2005). Neurodevelopment on route p63. *Neuron* 48, 707-9.

Nixon, R. A., Wegiel, J., Kumar, A., Yu, W. H., Peterhoff, C., Cataldo, A. and Cuervo, A. M. (2005). Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. *J Neuropathol Exp Neurol* 64, 113-22.

Nixon, R. A. and Yang, D. S. (2011). Autophagy failure in Alzheimer's disease--locating the primary defect. *Neurobiol Dis* 43, 38-45.

Nunes, A. F., Amaral, J. D., Lo, A. C., Fonseca, M. B., Viana, R. J., Callaerts-Vegh, Z., D'Hooge, R. and Rodrigues, C. M. (2012). TUDCA, a bile acid, attenuates amyloid precursor protein processing and amyloid-β deposition in APP/PS1 mice. *Mol Neurobiol* 45, 440-54.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T. and Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* 288, 1053-8.

Ohsawa, S., Hamada, S., Kuida, K., Yoshida, H., Igaki, T. and Miura, M. (2010). Maturation of the olfactory sensory neurons by Apaf-1/caspase-9-mediated caspase activity. *Proc Natl Acad Sci U S A* 107, 13366-71.

Ohyagi, Y., Asahara, H., Chui, D. H., Tsuruta, Y., Sakae, N., Miyoshi, K., Yamada, T., Kikuchi, H., Taniwaki, T., Murai, H. et al. (2005). Intracellular Abeta42 activates p53 promoter: a pathway to neurodegeneration in Alzheimer's disease. *FASEB J* 19, 255-7.

Oppenheim, R. W. (1991). Cell death during development of the nervous system. *Annu Rev Neurosci* 14, 453-501.

Oveson, B. C., Iwase, T., Hackett, S. F., Lee, S. Y., Usui, S., Sedlak, T. W., Snyder, S. H., Campochiaro, P. A. and Sung, J. U. (2011). Constituents of bile, bilirubin and TUDCA, protect against oxidative stress-induced retinal degeneration. *J Neurochem* 116, 144-53.

Papoutsaki, M., Moretti, F., Lanza, M., Marinari, B., Sartorelli, V., Guerrini, L., Chimenti, S., Levrero, M. and Costanzo, A. (2005). A p38-dependent pathway regulates DeltaNp63 DNA binding to p53-dependent promoters in UV-induced apoptosis of keratinocytes. *Oncogene* 24, 6970-5.

Parihar, M. S. and Brewer, G. J. (2010). Amyloid-β as a modulator of synaptic plasticity. *J Alzheimers Dis* 22, 741-63.

Paris, M., Rouleau, M., Pucéat, M. and Aberdam, D. (2012). Regulation of skin aging and heart development by TAp63. *Cell Death Differ* 19, 186-93.

Paumgartner, G. and Beuers, U. (2002). Ursodeoxycholic acid in cholestatic liver disease: mechanisms of action and therapeutic use revisited. *Hepatology* 36, 525-31.

Pearson, H. A. and Peers, C. (2006). Physiological roles for amyloid beta peptides. *J Physiol* 575, 5-10.

Pereira, J. D., Sansom, S. N., Smith, J., Dobenecker, M. W., Tarakhovsky, A. and Livesey, F. J. Ezh2, the histone methyltransferase of PRC2, regulates the balance between self-renewal and differentiation in the cerebral cortex. *Proc Natl Acad Sci U S A* 107, 15957-62.

Phillips, M. J., Walker, T. A., Choi, H. Y., Faulkner, A. E., Kim, M. K., Sidney, S. S., Boyd, A. P., Nickerson, J. M., Boatright, J. H. and Pardue, M. T. (2008). Tauroursodeoxycholic acid preservation of photoreceptor structure and function in the rd10 mouse through postnatal day 30. *Invest Ophthalmol Vis Sci* 49, 2148-55.

Pietsch, E. C., Perchiniak, E., Canutescu, A. A., Wang, G., Dunbrack, R. L. and Murphy, M. E. (2008). Oligomerization of BAK by p53 utilizes conserved residues of the p53 DNA binding domain. *J Biol Chem* 283, 21294-304.

Pignatelli, A., Ackman, J. B., Vigetti, D., Beltrami, A. P., Zucchini, S. and Belluzzi, O. (2009). A potential reservoir of immature dopaminergic replacement neurons in the adult mammalian olfactory bulb. *Pflugers Arch* 457, 899-915.

Pike, C. J., Burdick, D., Walencewicz, A. J., Glabe, C. G. and Cotman, C. W. (1993). Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. *J Neurosci* 13, 1676-87.

Pike, C. J., Walencewicz, A. J., Glabe, C. G. and Cotman, C. W. (1991). Aggregation-related toxicity of synthetic beta-amyloid protein in hippocampal cultures. *Eur J Pharmacol* 207, 367-8.

Pillot, T., Drouet, B., Queillé, S., Labeur, C., Vandekerchkhove, J., Rosseneu, M., Pinçon-Raymond, M. and Chambaz, J. (1999). The nonfibrillar amyloid beta-peptide induces apoptotic neuronal cell death: involvement of its C-terminal fusogenic domain. *J Neurochem* 73, 1626-34.

Plant, L. D., Boyle, J. P., Smith, I. F., Peers, C. and Pearson, H. A. (2003). The production of amyloid beta peptide is a critical requirement for the viability of central neurons. *J Neurosci* 23, 5531-5.

Pollard, S. M., Conti, L., Sun, Y., Goffredo, D. and Smith, A. (2006). Adherent Neural Stem (NS) Cells from Fetal and Adult Forebrain. *Cerebral Cortex* 16, i112-i120.

Porayette, P., Gallego, M. J., Kaltcheva, M. M., Bowen, R. L., Vadakkadath Meethal, S. and Atwood, C. S. (2009). Differential processing of amyloid-beta precursor protein directs human embryonic stem cell proliferation and differentiation into neuronal precursor cells. *J Biol Chem* 284, 23806-17.

Pozniak, C. D., Barnabé-Heider, F., Rymar, V. V., Lee, A. F., Sadikot, A. F. and Miller, F. D. (2002). p73 is required for survival and maintenance of CNS neurons. *J Neurosci* 22, 9800-9.

Pozniak, C. D., Radinovic, S., Yang, A., McKeon, F., Kaplan, D. R. and Miller, F. D. (2000). An anti-apoptotic role for the p53 family member, p73, during developmental neuron death. *Science* 289, 304-6.

Pradelli, L. A., Bénéteau, M. and Ricci, J. E. (2010). Mitochondrial control of caspase-dependent and -independent cell death. *Cell Mol Life Sci* 67, 1589-97.

Pratt, T., Sharp, L., Nichols, J., Price, D. J. and Mason, J. O. (2000). Embryonic Stem Cells and Transgenic Mice Ubiquitously Expressing a Tau-Tagged Green Fluorescent Protein. *Developmental Biology* 228, 19-28.

Pua, H. H., Dzhagalov, I., Chuck, M., Mizushima, N. and He, Y. W. (2007). A critical role for the autophagy gene Atg5 in T cell survival and proliferation. *J Exp Med* 204, 25-31.

Putcha, G. V., Deshmukh, M. and Johnson, E. M. (2000). Inhibition of apoptotic signaling cascades causes loss of trophic factor dependence during neuronal maturation. *J Cell Biol* 149, 1011-8.

Ramalho, R. M., Borralho, P. M., Castro, R. E., Solá, S., Steer, C. J. and Rodrigues, C. M. (2006). Tauroursodeoxycholic acid modulates p53-mediated apoptosis in Alzheimer's disease mutant neuroblastoma cells. *J Neurochem* 98, 1610-8.

Ramalho, R. M., Nunes, A. F., Dias, R. B., Amaral, J. D., Lo, A. C., D'Hooge, R., Sebastião, A. M. and Rodrigues, C. M. (2013). Tauroursodeoxycholic acid suppresses amyloid β-induced synaptic toxicity in vitro and in APP/PS1 mice. *Neurobiol Aging* 34, 551-61.

Ramalho, R. M., Ribeiro, P. S., Solá, S., Castro, R. E., Steer, C. J. and Rodrigues, C. M. (2004). Inhibition of the E2F-1/p53/Bax pathway by tauroursodeoxycholic acid in amyloid beta-peptide-induced apoptosis of PC12 cells. *Journal of neurochemistry* 90, 567-75.

Ramalho, R. M., Viana, R. J., Low, W. C., Steer, C. J. and Rodrigues, C. M. (2008). Bile acids and apoptosis modulation: an emerging role in experimental Alzheimer's disease. *Trends Mol Med* 14, 54-62.

Ramsden, M., Henderson, Z. and Pearson, H. A. (2002). Modulation of Ca2+ channel currents in primary cultures of rat cortical neurones by amyloid beta protein (1-40) is dependent on solubility status. *Brain Res* 956, 254-61.

Ramsden, M., Plant, L. D., Webster, N. J., Vaughan, P. F., Henderson, Z. and Pearson, H. A. (2001). Differential effects of unaggregated and aggregated amyloid beta protein (1-40) on K(+) channel currents in primary cultures of rat cerebellar granule and cortical neurones. *J Neurochem* 79, 699-712.

Ravikumar, B. and Rubinsztein, D. C. (2004). Can autophagy protect against neurodegeneration caused by aggregate-prone proteins? *Neuroreport* 15, 2443-5.

Ray, P. D., Huang, B. W. and Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal* 24, 981-90.

Raynaud, F. and Marcilhac, A. (2006). Implication of calpain in neuronal apoptosis. A possible regulation of Alzheimer's disease. *FEBS J* 273, 3437-43.

Reynolds, B. A. and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255, 1707-10.

Reynolds, B. A. and Weiss, S. (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev Biol* 175, 1-13.

Robles, A. I., Bemmels, N. A., Foraker, A. B. and Harris, C. C. (2001). APAF-1 is a transcriptional target of p53 in DNA damage-induced apoptosis. *Cancer Res* 61, 6660-4.

Rocco, J. W. and Ellisen, L. W. (2006). p63 and p73: life and death in squamous cell carcinoma. *Cell Cycle* 5, 936-40.

Rodrigues, C. M., Fan, G., Ma, X., Kren, B. T. and Steer, C. J. (1998). A novel role for ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation. *J Clin Invest* 101, 2790-9.

Rodrigues, C. M., Solá, S., Nan, Z., Castro, R. E., Ribeiro, P. S., Low, W. C. and Steer, C. J. (2003a). Tauroursodeoxycholic acid reduces apoptosis and protects against neurological injury after acute hemorrhagic stroke in rats. *Proc Natl Acad Sci U S A* 100, 6087-92.

Rodrigues, C. M., Solá, S. and Brites, D. (2002a). Bilirubin induces apoptosis via the mitochondrial pathway in developing rat brain neurons. *Hepatology* 35, 1186-95.

Rodrigues, C. M., Solá, S., Brito, M. A., Brondino, C. D., Brites, D. and Moura, J. J. (2001). Amyloid beta-peptide disrupts mitochondrial membrane lipid and protein structure: protective role of tauroursodeoxycholate. *Biochem Biophys Res Commun* 281, 468-74.

Rodrigues, C. M., Solá, S., Sharpe, J. C., Moura, J. J. and Steer, C. J. (2003b). Tauroursodeoxycholic acid prevents Bax-induced membrane perturbation and cytochrome C release in isolated mitochondria. *Biochemistry* 42, 3070-80.

Rodrigues, C. M., Solá, S., Silva, R. and Brites, D. (2000a). Bilirubin and amyloid-beta peptide induce cytochrome c release through mitochondrial membrane permeabilization. *Mol Med* 6, 936-46.

Rodrigues, C. M., Spellman, S. R., Solá, S., Grande, A. W., Linehan-Stieers, C., Low, W. C. and Steer, C. J. (2002b). Neuroprotection by a bile acid in an acute stroke model in the rat. *J Cereb Blood Flow Metab* 22, 463-71.

Rodrigues, C. M., Stieers, C. L., Keene, C. D., Ma, X., Kren, B. T., Low, W. C. and Steer, C. J. (2000b). Tauroursodeoxycholic acid partially prevents apoptosis induced by 3-nitropropionic acid: evidence for a mitochondrial pathway independent of the permeability transition. *J Neurochem* 75, 2368-79.

Roselli, F., Tirard, M., Lu, J., Hutzler, P., Lamberti, P., Livrea, P., Morabito, M. and Almeida, O. F. (2005). Soluble beta-amyloid1-40 induces NMDA-dependent degradation of postsynaptic density-95 at glutamatergic synapses. *J Neurosci* 25, 11061-70.

Sabapathy, K., Jochum, W., Hochedlinger, K., Chang, L., Karin, M. and Wagner, E. F. (1999). Defective neural tube morphogenesis and altered apoptosis in the absence of both JNK1 and JNK2. *Mech Dev* 89, 115-24.

Sah, V. P., Attardi, L. D., Mulligan, G. J., Williams, B. O., Bronson, R. T. and Jacks, T. (1995). A subset of p53-deficient embryos exhibit exencephaly. *Nat Genet* 10, 175-80.

Santos, D. M., Santos, M. M., Moreira, R., Solá, S. and Rodrigues, C. M. (2012a). Synthetic Condensed 1,4-naphthoquinone Derivative Shifts Neural Stem Cell Differentiation by Regulating Redox State. *Mol Neurobiol*.

Santos, D. M., Xavier, J. M., Morgado, A. L., Solá, S. and Rodrigues, C. M. (2012b). Distinct Regulatory Functions of Calpain 1 and 2 during Neural Stem Cell Self-Renewal and Differentiation. *PLoS One* 7, e33468.

Sarkar, S. A. and Sharma, R. P. (2002). Expression of selected apoptosis related genes, MIF, IGIF and TNF alpha, during retinoic acid-induced neural differentiation in murine embryonic stem cells. *Cell Struct Funct* 27, 99-107.

Sato, N., Hotta, K., Waguri, S., Nitatori, T., Tohyama, K., Tsujimoto, Y. and Uchiyama, Y. (1994). Neuronal differentiation of PC12 cells as a result of prevention of cell death by bcl-2. *J Neurobiol* 25, 1227-34.

Sayan, B. S., Sayan, A. E., Yang, A. L., Aqeilan, R. I., Candi, E., Cohen, G. M., Knight, R. A., Croce, C. M. and Melino, G. (2007b). Cleavage of the transactivation-inhibitory domain of p63 by caspases enhances apoptosis. *Proc Natl Acad Sci U S A* 104, 10871-6.

Schliebs, R. (2005). Basal forebrain cholinergic dysfunction in Alzheimer's disease--interrelationship with beta-amyloid, inflammation and neurotrophin signaling. *Neurochem Res* 30, 895-908.

Schoemaker, M. H., Conde de la Rosa, L., Buist-Homan, M., Vrenken, T. E., Havinga, R., Poelstra, K., Haisma, H. J., Jansen, P. L. and Moshage, H. (2004). Tauroursodeoxycholic acid protects rat hepatocytes from bile acid-induced apoptosis via activation of survival pathways. *Hepatology* 39, 1563-73.

Schwerk, C. and Schulze-Osthoff, K. (2003). Non-apoptotic functions of caspases in cellular proliferation and differentiation. *Biochem Pharmacol* 66, 1453-8.

Scoumanne, A. and Chen, X. (2008). Protein methylation: a new mechanism of p53 tumor suppressor regulation. *Histol Histopathol* 23, 1143-9.

Selkoe, D. J. (2001b). Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* 81, 741-66.

Selkoe, D. J. and Wolfe, M. S. (2007). Presenilin: running with scissors in the membrane. *Cell* 131, 215-21.

Seri, B., García-Verdugo, J. M., McEwen, B. S. and Alvarez-Buylla, A. (2001). Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J Neurosci* 21, 7153-60.

Setchell, K. D., Bragetti, P., Zimmer-Nechemias, L., Daugherty, C., Pelli, M. A., Vaccaro, R., Gentili, G., Distrutti, E., Dozzini, G. and Morelli, A. (1992). Oral bile acid treatment and the patient with Zellweger syndrome. *Hepatology* 15, 198-207.

Shankar, G. M., Bloodgood, B. L., Townsend, M., Walsh, D. M., Selkoe, D. J. and Sabatini, B. L. (2007). Natural oligomers of the Alzheimer amyloid-beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. *J Neurosci* 27, 2866-75.

Shaulian, E. and Karin, M. (2002). AP-1 as a regulator of cell life and death. *Nat Cell Biol* 4, E131-6.

She, Q. B., Huang, C., Zhang, Y. and Dong, Z. (2002). Involvement of c-jun NH(2)-terminal kinases in resveratrol-induced activation of p53 and apoptosis. *Mol Carcinog* 33, 244-50.

Shibata, T., Yamada, K., Watanabe, M., Ikenaka, K., Wada, K., Tanaka, K. and Inoue, Y. (1997). Glutamate transporter GLAST is expressed in the radial glia-astrocyte lineage of developing mouse spinal cord. *J Neurosci* 17, 9212-9.

Shimizu, S., Kanaseki, T., Mizushima, N., Mizuta, T., Arakawa-Kobayashi, S., Thompson, C. B. and Tsujimoto, Y. (2004). Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. *Nat Cell Biol* 6, 1221-8.

Silva, J., Chambers, I., Pollard, S. and Smith, A. (2006). Nanog promotes transfer of pluripotency after cell fusion. *Nature* 441, 997-1001.

Sims, R. J. and Reinberg, D. (2008). Is there a code embedded in proteins that is based on post-translational modifications? *Nat Rev Mol Cell Biol* 9, 815-20.

Singh, R., Xiang, Y., Wang, Y., Baikati, K., Cuervo, A. M., Luu, Y. K., Tang, Y., Pessin, J. E., Schwartz, G. J. and Czaja, M. J. (2009). Autophagy regulates adipose mass and differentiation in mice. *J Clin Invest* 119, 3329-39.

Slack, R. S., Belliveau, D. J., Rosenberg, M., Atwal, J., Lochmüller, H., Aloyz, R., Haghighi, A., Lach, B., Seth, P., Cooper, E. et al. (1996). Adenovirus-mediated gene transfer of the tumor suppressor, p53, induces apoptosis in postmitotic neurons. *J Cell Biol* 135, 1085-96.

Smale, G., Nichols, N. R., Brady, D. R., Finch, C. E. and Horton, W. E. (1995). Evidence for apoptotic cell death in Alzheimer's disease. *Exp Neurol* 133, 225-30.

Smith, J., Ladi, E., Mayer-Proschel, M. and Noble, M. (2000). Redox state is a central modulator of the balance between self-renewal and differentiation in a dividing glial precursor cell. *Proc Natl Acad Sci U S A* 97, 10032-7.

Solá, S., Castro, R. E., Laires, P. A., Steer, C. J. and Rodrigues, C. M. (2003a). Tauroursodeoxycholic acid prevents amyloid-beta peptide-induced neuronal death via a phosphatidylinositol 3-kinase-dependent signaling pathway. *Molecular medicine* 9, 226-34.

Solá, S., Ma, X., Castro, R. E., Kren, B. T., Steer, C. J. and Rodrigues, C. M. (2003b). Ursodeoxycholic acid modulates E2F-1 and p53 expression through a caspase-independent mechanism in transforming growth factor beta1-induced apoptosis of rat hepatocytes. *J Biol Chem* 278, 48831-8.

Solá, S., Amaral, J. D., Borralho, P. M., Ramalho, R. M., Castro, R. E., Aranha, M. M., Steer, C. J. and Rodrigues, C. M. (2006). Functional modulation of nuclear steroid receptors by tauroursodeoxycholic acid reduces amyloid beta-peptide-induced apoptosis. *Mol Endocrinol* 20, 2292-303.

Solá, S., Amaral, J. D., Castro, R. E., Ramalho, R. M., Borralho, P. M., Kren, B. T., Tanaka, H., Steer, C. J. and Rodrigues, C. M. (2005). Nuclear translocation of UDCA by the glucocorticoid receptor is required to reduce TGF-beta1-induced apoptosis in rat hepatocytes. *Hepatology* 42, 925-34.

Solá, S., Aranha, M. M. and Rodrigues, C. M. (2012). Driving apoptosis-relevant proteins toward neural differentiation. *Mol Neurobiol* 46, 316-31.

Solá, S., Castro, R. E., Kren, B. T., Steer, C. J. and Rodrigues, C. M. (2004). Modulation of nuclear steroid receptors by ursodeoxycholic acid inhibits TGF-beta1-induced E2F-1/p53-mediated apoptosis of rat hepatocytes. *Biochemistry* 43, 8429-38.

Solá, S., Castro, R. E., Laires, P. A., Steer, C. J. and Rodrigues, C. M. (2003). Tauroursodeoxycholic acid prevents amyloid-beta peptide-induced neuronal death via a phosphatidylinositol 3-kinase-dependent signaling pathway. *Mol Med* 9, 226-34.

Solá, S., Morgado, A. L. and Rodrigues, C. M. (2013). Death receptors and mitochondria: two prime triggers of neural apoptosis and differentiation. *Biochim Biophys Acta* 1830, 2160-6.

Solá, S., Xavier, J. M., Santos, D. M., Aranha, M. M., Morgado, A. L., Jepsen, K. and Rodrigues, C. M. (2011). p53 interaction with JMJD3 results in its nuclear distribution during mouse neural stem cell differentiation. *PLoS One* 6, e18421.

Song, G., Chen, G. G., Yun, J. P. and Lai, P. B. (2009). Association of p53 with Bid induces cell death in response to etoposide treatment in hepatocellular carcinoma. *Curr Cancer Drug Targets* 9, 871-80.

Song, H. and Xu, Y. (2007). Gain of function of p53 cancer mutants in disrupting critical DNA damage response pathways. *Cell Cycle* 6, 1570-3.

Sorenson, C. M. (2004). Bcl-2 family members and disease. *Biochim Biophys Acta* 1644, 169-77.

Sotthibundhu, A., Li, Q. X., Thangnipon, W. and Coulson, E. J. (2009). Abeta(1-42) stimulates adult SVZ neurogenesis through the p75 neurotrophin receptor. *Neurobiol Aging* 30, 1975-85.

Spassky, N., Merkle, F. T., Flames, N., Tramontin, A. D., García-Verdugo, J. M. and Alvarez-Buylla, A. (2005). Adult ependymal cells are postmitotic and are derived from radial glial cells during embryogenesis. *J Neurosci* 25, 10-8.

Spiliotopoulos, D., Goffredo, D., Conti, L., Di Febo, F., Biella, G., Toselli, M. and Cattaneo, E. (2009). An optimized experimental strategy for efficient conversion of embryonic stem (ES)-derived mouse neural stem (NS) cells into a nearly homogeneous mature neuronal population. *Neurobiol Dis* 34, 320-31.

Spillantini, M. G. and Goedert, M. (1998). Tau protein pathology in neurodegenerative diseases. *Trends Neurosci* 21, 428-33.

Spradling, A., Drummond-Barbosa, D. and Kai, T. (2001). Stem cells find their niche. *Nature* 414, 98-104.

Stanfield, B. B. and Trice, J. E. (1988). Evidence that granule cells generated in the dentate gyrus of adult rats extend axonal projections. *Exp Brain Res* 72, 399-406.

Stehmeier, P. and Muller, S. (2009). Regulation of p53 family members by the ubiquitin-like SUMO system. *DNA Repair (Amst)* 8, 491-8.

Steiner, H. and Haass, C. (2000). Intramembrane proteolysis by presenilins. *Nat Rev Mol Cell Biol* 1, 217-24.

Suganuma, T., Mushegian, A., Swanson, S. K., Abmayr, S. M., Florens, L., Washburn, M. P. and Workman, J. L. (2010). The ATAC acetyltransferase complex coordinates MAP kinases to regulate JNK target genes. *Cell* 142, 726-36.

Suzuki, A. and Tsutomi, Y. (1998). Bcl-2 accelerates the neuronal differentiation: new evidence approaching to the biofunction of bcl-2 in the neuronal system. *Brain Res* 801, 59-66.

Talos, F., Abraham, A., Vaseva, A. V., Holembowski, L., Tsirka, S. E., Scheel, A., Bode, D., Dobbelstein, M., Brück, W. and Moll, U. M. (2010). p73 is an essential regulator of neural stem cell maintenance in embryonal and adult CNS neurogenesis. *Cell Death Differ* 17, 1816-29.

Tamaoka, A., Sawamura, N., Fukushima, T., Shoji, S., Matsubara, E., Shoji, M., Hirai, S., Furiya, Y., Endoh, R. and Mori, H. (1997). Amyloid beta protein 42(43) in cerebrospinal fluid of patients with Alzheimer's disease. *J Neurol Sci* 148, 41-5.

Tang, T. S., Slow, E., Lupu, V., Stavrovskaya, I. G., Sugimori, M., Llinás, R., Kristal, B. S., Hayden, M. R. and Bezprozvanny, I. (2005). Disturbed Ca2+ signaling and apoptosis of medium spiny neurons in Huntington's disease. *Proc Natl Acad Sci U S A* 102, 2602-7.

Tanida, I., Nishitani, T., Nemoto, T., Ueno, T. and Kominami, E. (2002). Mammalian Apg12p, but not the Apg12p.Apg5p conjugate, facilitates LC3 processing. *Biochem Biophys Res Commun* 296, 1164-70.

Tanida, I., Tanida-Miyake, E., Ueno, T. and Kominami, E. (2001). The human homolog of Saccharomyces cerevisiae Apg7p is a Protein-activating enzyme for multiple substrates including human Apg12p, GATE-16, GABARAP, and MAP-LC3. *J Biol Chem* 276, 1701-6.

Tedeschi, A. and Di Giovanni, S. (2009). The non-apoptotic role of p53 in neuronal biology: enlightening the dark side of the moon. *EMBO Rep* 10, 576-83.

Temple, S. and Alvarez-Buylla, A. (1999). Stem cells in the adult mammalian central nervous system. *Curr Opin Neurobiol* 9, 135-41.

Testoni, B., Borrelli, S., Tenedini, E., Alotto, D., Castagnoli, C., Piccolo, S., Tagliafico, E., Ferrari, S., Viganò, M. A. and Mantovani, R. (2006). Identification of new p63 targets in human keratinocytes. *Cell Cycle* 5, 2805-11.

Thinakaran, G. and Koo, E. H. (2008). Amyloid precursor protein trafficking, processing, and function. *J Biol Chem* 283, 29615-9.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S. and Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145-7.

Thorburn, A. (2008). Apoptosis and autophagy: regulatory connections between two supposedly different processes. *Apoptosis* 13, 1-9.

Tissir, F., Ravni, A., Achouri, Y., Riethmacher, D., Meyer, G. and Goffinet, A. M. (2009). DeltaNp73 regulates neuronal survival in vivo. *Proc Natl Acad Sci U S A* 106, 16871-6.

Toh, W. H., Siddique, M. M., Boominathan, L., Lin, K. W. and Sabapathy, K. (2004). c-Jun regulates the stability and activity of the p53 homologue, p73. *J Biol Chem* 279, 44713-22.

Tomasini, R., Tsuchihara, K., Wilhelm, M., Fujitani, M., Rufini, A., Cheung, C. C., Khan, F., Itie-Youten, A., Wakeham, A., Tsao, M. S. et al. (2008). TAp73 knockout shows genomic instability with infertility and tumor suppressor functions. *Genes Dev* 22, 2677-91.

Toni, N., Teng, E. M., Bushong, E. A., Aimone, J. B., Zhao, C., Consiglio, A., van Praag, H., Martone, M. E., Ellisman, M. H. and Gage, F. H. (2007). Synapse formation on neurons born in the adult hippocampus. *Nat Neurosci* 10, 727-34.

Tooze, S. A. and Razi, M. (2009). The essential role of early endosomes in autophagy is revealed by loss of COPI function. *Autophagy* 5, 874-5.

Tsai, W. W., Nguyen, T. T., Shi, Y. and Barton, M. C. (2008). p53-targeted LSD1 functions in repression of chromatin structure and transcription in vivo. *Mol Cell Biol* 28, 5139-46.

Tseng, B. P., Green, K. N., Chan, J. L., Blurton-Jones, M. and LaFerla, F. M. (2008). Abeta inhibits the proteasome and enhances amyloid and tau accumulation. *Neurobiol Aging* 29, 1607-18.

Tsukamoto, S., Kuma, A. and Mizushima, N. (2008). The role of autophagy during the oocyte-to-embryo transition. *Autophagy* 4, 1076-8.

Uberti, D., Piccioni, L., Cadei, M., Grigolato, P., Rotter, V. and Memo, M. (2001). p53 is dispensable for apoptosis but controls neurogenesis of mouse dentate gyrus cells following gamma-irradiation. *Brain Res Mol Brain Res* 93, 81-9.

van Praag, H., Schinder, A. F., Christie, B. R., Toni, N., Palmer, T. D. and Gage, F. H. (2002). Functional neurogenesis in the adult hippocampus. *Nature* 415, 1030-4.

Vanbokhoven, H., Melino, G., Candi, E. and Declercq, W. (2011). p63, a story of mice and men. *The Journal of investigative dermatology* 131, 1196-207.

Vaseva, A. V., Marchenko, N. D. and Moll, U. M. (2009). The transcription-independent mitochondrial p53 program is a major contributor to nutlin-induced apoptosis in tumor cells. *Cell Cycle* 8, 1711-9.

Vessoni, A. T., Muotri, A. R. and Okamoto, O. K. (2012). Autophagy in stem cell maintenance and differentiation. *Stem Cells Dev* 21, 513-20.

Viana, R. J., Ramalho, R. M., Nunes, A. F., Steer, C. J. and Rodrigues, C. M. (2010). Modulation of amyloid-beta peptide-induced toxicity through inhibition of JNK nuclear localization and caspase-2 activation. *Journal of Alzheimer's disease : JAD* 22, 557-68.

Viana, R. J., Steer, C. J. and Rodrigues, C. M. (2011). Amyloid-beta Peptide-Induced Secretion of Endoplasmic Reticulum Chaperone Glycoprotein GRP94. *Journal of Alzheimer's disease : JAD*.

Vousden, K. H. and Lu, X. (2002). Live or let die: the cell's response to p53. *Nat Rev Cancer* 2, 594-604.

Vázquez, M. C., Vargas, L. M., Inestrosa, N. C. and Alvarez, A. R. (2009). c-Abl modulates AICD dependent cellular responses: transcriptional induction and apoptosis. *J Cell Physiol* 220, 136-43.

Vázquez, P., Arroba, A. I., Cecconi, F., de la Rosa, E. J., Boya, P. and de Pablo, F. (2012). Atg5 and Ambra1 differentially modulate neurogenesis in neural stem cells. *Autophagy* 8, 187-99.

Walsh, G. S., Orike, N., Kaplan, D. R. and Miller, F. D. (2004). The invulnerability of adult neurons: a critical role for p73. *J Neurosci* 24, 9638-47.

Wang, H., Ma, J., Tan, Y., Wang, Z., Sheng, C., Chen, S. and Ding, J. (2010). Amyloid-beta1-42 induces reactive oxygen species-mediated autophagic cell death in U87 and SH-SY5Y cells. *J Alzheimers Dis* 21, 597-610.

Wang, R., Dineley, K. T., Sweatt, J. D. and Zheng, H. (2004). Presenilin 1 familial Alzheimer's disease mutation leads to defective associative learning and impaired adult neurogenesis. *Neuroscience* 126, 305-12.

Wang, S., Rosengren, L., Hamberger, A. and Haglid, K. (2001). Antisense inhibition of BCL-2 expression induces retinoic acid-mediated cell death during differentiation of human NT2N neurons. *J Neurochem* 76, 1089-98.

Watson, A., Eilers, A., Lallemand, D., Kyriakis, J., Rubin, L. L. and Ham, J. (1998). Phosphorylation of c-Jun is necessary for apoptosis induced by survival signal withdrawal in cerebellar granule neurons. *J Neurosci* 18, 751-62.

Watson, K. and Fan, G. H. (2005). Macrophage inflammatory protein 2 inhibits beta-amyloid peptide (1-42)-mediated hippocampal neuronal apoptosis through activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways. *Mol Pharmacol* 67, 757-65.

Watt, F. M. and Hogan, B. L. (2000). Out of Eden: stem cells and their niches. *Science* 287, 1427-30.

Weiss, S., Reynolds, B. A., Vescovi, A. L., Morshead, C., Craig, C. G. and van der Kooy, D. (1996). Is there a neural stem cell in the mammalian forebrain? *Trends Neurosci* 19, 387-93.

Wen, P. H., Hof, P. R., Chen, X., Gluck, K., Austin, G., Younkin, S. G., Younkin, L. H., DeGasperi, R., Gama Sosa, M. A., Robakis, N. K. et al. (2004). The presenilin-1 familial Alzheimer disease mutant P117L impairs neurogenesis in the hippocampus of adult mice. *Exp Neurol* 188, 224-37.

Westfall, M. D., Joyner, A. S., Barbieri, C. E., Livingstone, M. and Pietenpol, J. A. (2005). Ultraviolet radiation induces phosphorylation and ubiquitin-mediated degradation of DeltaNp63alpha. *Cell cycle* 4, 710-6.

Wetzel, M. K., Naska, S., Laliberté, C. L., Rymar, V. V., Fujitani, M., Biernaskie, J. A., Cole, C. J., Lerch, J. P., Spring, S., Wang, S. H. et al. (2008). p73 regulates neurodegeneration and phospho-tau accumulation during aging and Alzheimer's disease. *Neuron* 59, 708-21.

Whitfield, J., Neame, S. J., Paquet, L., Bernard, O. and Ham, J. (2001). Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. *Neuron* 29, 629-43.

Whitmarsh, A. J. and Davis, R. J. (1996). Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med (Berl)* 74, 589-607.

Whitson, J. S., Glabe, C. G., Shintani, E., Abcar, A. and Cotman, C. W. (1990). Beta-amyloid protein promotes neuritic branching in hippocampal cultures. *Neurosci Lett* 110, 319-24.

Whitson, J. S., Selkoe, D. J. and Cotman, C. W. (1989). Amyloid beta protein enhances the survival of hippocampal neurons in vitro. *Science* 243, 1488-90.

Wilhelm, M. T., Rufini, A., Wetzel, M. K., Tsuchihara, K., Inoue, S., Tomasini, R., Itie-Youten, A., Wakeham, A., Arsenian-Henriksson, M., Melino, G. et al. (2010). Isoform-specific p73 knockout mice reveal a novel role for delta Np73 in the DNA damage response pathway. *Genes Dev* 24, 549-60.

Wirawan, E., Vanden Berghe, T., Lippens, S., Agostinis, P. and Vandenabeele, P. (2012). Autophagy: for better or for worse. *Cell Res* 22, 43-61.

Wong, E. and Cuervo, A. M. (2010). Autophagy gone awry in neurodegenerative diseases. *Nat Neurosci* 13, 805-11.

Wong, K., Zhang, J., Awasthi, S., Sharma, A., Rogers, L., Matlock, E. F., Van Lint, C., Karpova, T., McNally, J. and Harrod, R. (2004). Nerve growth factor receptor signaling induces histone acetyltransferase domain-dependent nuclear translocation of p300/CREB-binding protein-associated factor and hGCN5 acetyltransferases. *J Biol Chem* 279, 55667-74.

Wu, G., Nomoto, S., Hoque, M. O., Dracheva, T., Osada, M., Lee, C. C., Dong, S. M., Guo, Z., Benoit, N., Cohen, Y. et al. (2003). DeltaNp63alpha and TAp63alpha regulate transcription of genes with distinct biological functions in cancer and development. *Cancer Res* 63, 2351-7.

Wu, H. and Sun, Y. E. (2006). Epigenetic regulation of stem cell differentiation. *Pediatr Res* 59, 21R-5R.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. and Greenberg, M. E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 270, 1326-31.

Xie, Z. and Klionsky, D. J. (2007). Autophagosome formation: core machinery and adaptations. *Nat Cell Biol* 9, 1102-9.

Xu, Y. (2003). Regulation of p53 responses by post-translational modifications. *Cell Death Differ* 10, 400-3.

Yang, A., Kaghad, M., Caput, D. and McKeon, F. (2002). On the shoulders of giants: p63, p73 and the rise of p53. *Trends Genet* 18, 90-5.

Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dötsch, V., Andrews, N. C., Caput, D. and McKeon, F. (1998). p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell* 2, 305-16.

Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R. T., Tabin, C., Sharpe, A., Caput, D., Crum, C. et al. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 398, 714-8.

Yang, A., Walker, N., Bronson, R., Kaghad, M., Oosterwegel, M., Bonnin, J., Vagner, C., Bonnet, H., Dikkes, P., Sharpe, A. et al. (2000). p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature* 404, 99-103.

Yang, L., Ye, C. Y., Huang, X. T., Tang, X. C. and Zhang, H. Y. (2012). Decreased accumulation of subcellular amyloid-β with improved mitochondrial function mediates the neuroprotective effect of huperzine A. *J Alzheimers Dis* 31, 131-42.

Yang, Z. and Klionsky, D. J. (2010). Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol* 22, 124-31.

Yankner, B. A., Duffy, L. K. and Kirschner, D. A. (1990). Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. *Science* 250, 279-82.

Yao, J., Irwin, R. W., Zhao, L., Nilsen, J., Hamilton, R. T. and Brinton, R. D. (2009). Mitochondrial bioenergetic deficit precedes Alzheimer's pathology in female mouse model of Alzheimer's disease. *Proc Natl Acad Sci U S A* 106, 14670-5.

Yao, J. Y., Pao, C. C. and Chen, J. K. (2010a). Transcriptional activity of TAp63 promoter is regulated by c-jun. *Journal of cellular physiology* 225, 898-904.

Yao, M., Nguyen, T. V. and Pike, C. J. (2005). Beta-amyloid-induced neuronal apoptosis involves c-Jun N-terminal kinase-dependent downregulation of Bcl-w. *J Neurosci* 25, 1149-58.

Yao, Z. and Wood, N. W. (2009). Cell death pathways in Parkinson's disease: role of mitochondria. *Antioxid Redox Signal* 11, 2135-49.

Yao, Z. G., Liu, Y., Zhang, L., Huang, L., Ma, C. M., Xu, Y. F., Zhu, H. and Qin, C. (2012). Co-location of HDAC2 and insulin signaling components in the adult mouse hippocampus. *Cell Mol Neurobiol* 32, 1337-42.

Ying, H., Chang, D. L., Zheng, H., McKeon, F. and Xiao, Z. X. (2005). DNA-binding and transactivation activities are essential for TAp63 protein degradation. *Mol Cell Biol* 25, 6154-64.

Youle, R. J. and Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* 9, 47-59.

Yu, L., Alva, A., Su, H., Dutt, P., Freundt, E., Welsh, S., Baehrecke, E. H. and Lenardo, M. J. (2004a). Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science* 304, 1500-2.

Yu, L., Lenardo, M. J. and Baehrecke, E. H. (2004b). Autophagy and caspases: a new cell death program. *Cell Cycle* 3, 1124-6.

Yu, L., Wan, F., Dutta, S., Welsh, S., Liu, Z., Freundt, E., Baehrecke, E. H. and Lenardo, M. (2006). Autophagic programmed cell death by selective catalase degradation. *Proc Natl Acad Sci U S A* 103, 4952-7.

Yu, W. H., Cuervo, A. M., Kumar, A., Peterhoff, C. M., Schmidt, S. D., Lee, J. H., Mohan, P. S., Mercken, M., Farmery, M. R., Tjernberg, L. O. et al. (2005). Macroautophagy--a novel Beta-amyloid peptide-generating pathway activated in Alzheimer's disease. *J Cell Biol* 171, 87-98.

Yu, W. H., Kumar, A., Peterhoff, C., Shapiro Kulnane, L., Uchiyama, Y., Lamb, B. T., Cuervo, A. M. and Nixon, R. A. (2004c). Autophagic vacuoles are enriched in amyloid precursor protein-secretase activities: implications for beta-amyloid peptide over-production and localization in Alzheimer's disease. *Int J Biochem Cell Biol* 36, 2531-40.

Yuan, J. and Yankner, B. A. (2000). Apoptosis in the nervous system. *Nature* 407, 802-9.

Yuan, Z. M., Shioya, H., Ishiko, T., Sun, X., Gu, J., Huang, Y. Y., Lu, H., Kharbanda, S., Weichselbaum, R. and Kufe, D. (1999). p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage. *Nature* 399, 814-7.

Zeng, M. and Zhou, J. N. (2008). Roles of autophagy and mTOR signaling in neuronal differentiation of mouse neuroblastoma cells. *Cell Signal* 20, 659-65.

Zhang, H., Wu, S. and Xing, D. (2011). YAP accelerates Aβ(25-35)-induced apoptosis through upregulation of Bax expression by interaction with p73. *Apoptosis* 16, 808-21.

Zhang, J., Yan, W. and Chen, X. (2006a). p53 is required for nerve growth factor-mediated differentiation of PC12 cells via regulation of TrkA levels. *Cell Death Differ* 13, 2118-28.

Zhang, K. Z., Westberg, J. A., Hölttä, E. and Andersson, L. C. (1996). BCL2 regulates neural differentiation. *Proc Natl Acad Sci U S A* 93, 4504-8.

Zhang, Q. B., Ji, X. Y., Huang, Q., Dong, J., Zhu, Y. D. and Lan, Q. (2006b). Differentiation profile of brain tumor stem cells: a comparative study with neural stem cells. *Cell Res* 16, 909-15.

Zhao, Y., Huang, Q., Yang, J., Lou, M., Wang, A., Dong, J., Qin, Z. and Zhang, T. (2010). Autophagy impairment inhibits differentiation of glioma stem/progenitor cells. *Brain Res* 1313, 250-8.

Zheng, H., Ying, H., Yan, H., Kimmelman, A. C., Hiller, D. J., Chen, A. J., Perry, S. R., Tonon, G., Chu, G. C., Ding, Z. et al. (2008). p53 and Pten control neural and glioma stem/progenitor cell renewal and differentiation. *Nature* 455, 1129-33.

Zheng, L., Marcusson, J. and Terman, A. (2006a). Oxidative stress and Alzheimer disease: the autophagy connection? *Autophagy* 2, 143-5.

Zheng, L., Roberg, K., Jerhammar, F., Marcusson, J. and Terman, A. (2006b). Oxidative stress induces intralysosomal accumulation of Alzheimer amyloid beta-protein in cultured neuroblastoma cells. *Ann N Y Acad Sci* 1067, 248-51.

Zhuang, W., Li, B., Long, L., Chen, L., Huang, Q. and Liang, Z. (2011). Induction of autophagy promotes differentiation of glioma-initiating cells and their radiosensitivity. *Int J Cancer* 129, 2720-31.