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Non-canonical roles of the anti-apoptotic protein Bcl-xL in the development of the central nervous system

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L'UNIVERSITE CLAUDE BERNARD LYON 1**

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**Non-canonical roles of the anti-apoptotic
protein Bcl-xL in the development of the
central nervous system**

Rôles non-canoniques de la protéine anti-apoptique Bcl-xL dans le développement
du système nerveux central

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MANDATORY FEATURES

Titre Français

Rôles non-canoniques de la protéine anti-apoptotique Bcl-xL dans le développement du système nerveux central

Mots-clés en Français

Protéines Bcl-2 - Bcl-xL- mitochondrie – caspase-3 - neurone – développement

Résumé en Français

Bcl-xL est une protéine anti-apoptotique de la famille Bcl-2, ayant une double localisation - mitochondrie et réticulum endoplasmique (RE). Elle est bien connue pour son effet antagoniste contre l'apoptose grâce à son interaction canonique avec Bax/Bak au niveau de la membrane externe de la mitochondrie. Très sensibles aux stress cellulaires, les neurones dépendent fortement de Bcl-xL pour leur survie au cours du développement.

L'activation non-létale de la caspase-3 a été décrite dans le neurone en développement et joue des fonctions physiologiques dans le développement des axones et la formation des synapses. Néanmoins, la manière dont les neurones activent et régulent ce signal, localement et temporellement, reste exclusive.

Dans un modèle de souris génétiquement modifié exprimant Bcl-xL exclusivement au niveau du RE généré par notre équipe, nous n'avons pas observé de mort cellulaire massive dans le système nerveux central (SNC) comme cela avait été précédemment rapporté chez les souris knock-out Bcl-xL. De manière inattendue, la caspase-3 active est abondante dans le cerveau mutant et la substance blanche de la moelle épinière. Les neurones dérivés de ces souris présentent une activation locale non létale de la caspase-3 dans l'axone, induisant une arborisation axonale et une maturation synaptique altérées, mais pas d'apoptose. Les anomalies du développement des neurones du SNC entraînent finalement une létalité périnatale,

potentiellement due à des perturbations des comportements d'alimentation et de déglutition. Nous démontrons également que l'activation de la caspase-3 induite par la perte de Bcl-xL mitochondrial est dépendante de Drp1.

Nos résultats suggèrent que le pool mitochondrial de Bcl-xL est nécessaire pour limiter l'activation locale de la caspase-3 à une intensité physiologique appropriée dans le SNC en développement. Cette découverte apporte un nouveau point de vue sur le rôle régulateur des protéines Bcl-2 dans la caspase-3 non létale physiologique et pathologique.

Titre Anglais

Non-canonical roles of the anti-apoptotic protein Bcl-xL in the development of the central nervous system

Mots-clés en Anglais

Bcl-2 proteins - Bcl-xL- mitochondria – caspase-3 - neuron - development

Résumé en Anglais

Bcl-xL is an anti-apoptotic protein of the Bcl-2 family, with a dual localization – mitochondria and endoplasmic reticulum (ER). It is well-known for its antagonizing effect against apoptosis due to its canonical interaction with Bax/Bak at the mitochondrial outer membrane. Highly sensitive to cellular stresses, neurons are dependent on Bcl-xL for survival over development.

Non-lethal caspase-3 activation has been described in the developing neuron and performs physiological functions in axon development and synapse formation. Notwithstanding, how neurons activate and regulate this signal, locally and temporally, remains exclusive.

In a genetically modified mouse model generated by our team that expresses Bcl-xL exclusively at the ER, we did not observe massive cell death in the central nervous system (CNS) as previously reported in Bcl-xL knockout (KO) mice. Unexpectedly, active caspase-3 was abundant in the mutant brain and spinal cord white matter. Neurons derived from these mice exhibit non-lethal local caspase-3 activation in the axon, inducing impaired axonal arborization

and synaptic maturation but not apoptosis. Anomalies in the development of CNS neurons ultimately caused perinatal lethality, potentially due to perturbations of feeding and swallowing behaviors. We further demonstrated that mitochondrial Bcl-xL loss-induced caspase-3 activation is related to dynamin-related protein 1 (Drp1).

Our results suggest that the mitochondrial pool of Bcl-xL is necessary to confine the local caspase-3 activation to appropriate physiological intensity in the developing CNS. This finding brings a novel point of view on the regulatory role of Bcl-2 proteins in the physiological and pathological non-lethal caspase-3.

Résumé substantiel en Français

L'apoptose est un type de mort cellulaire programmé jouant un rôle majeur en structurant la morphogénèse lors du développement des mammifères et en maintenant l'homéostasie des organismes multicellulaires. La perturbation de programme apoptotique est liée aux conditions pathologiques graves, y compris le cancer et les maladies neurodégénératives. Par conséquent, cette mort cellulaire intéresse grandement la communauté scientifique. L'apoptose s'initie par deux voies principales : la voie extrinsèque et la voie intrinsèque. La première se déclenche par l'activation des récepteurs de mort via des signaux extracellulaires. Celle-ci joue un rôle essentiel dans les fonctions du système immunitaire. La deuxième, également appelée la voie mitochondriale, répond aux stimuli intracellulaires et extracellulaires grâce à son lien avec la voie extrinsèque. Les deux voies apoptotiques déclenchent une cascade d'activation enzymatique qui active les protéines caspases qui sont considérées comme les exécuteurs de la mort cellulaire.

L'évènement-clé de la voie mitochondriale est la perméabilisation de la membrane extérieure de mitochondrie. Cela déclenche la libération de facteurs apoptogéniques au cytosol, y compris cytochrome c, et initie l'activation de caspase. La famille de protéine Bcl-2 gouverne l'intégrité de la membrane extérieure de mitochondrie et agit ainsi comme le chef régulateur de mort cellulaire par l'apoptose.

La famille de Bcl-2 est constituée de deux sous-groupes principaux : l'anti-apoptotique et le pro-apoptotique. Les protéines pro-apoptotiques ont la capacité de former les structures poreuses menant à la libération de cytochrome c. Ce dernier active les caspases et, par conséquent, l'apoptose. Les protéines anti-apoptotiques inhibent les pro-apoptotiques et conservent l'intégrité de mitochondrie.

A cause de la rapidité de la perméabilisation mitochondriale, cette étape a été considérée comme un évènement irréversible de l'apoptose. Néanmoins, il y a récemment des évidences qui montrent que l'activation de caspase-3, le principal « réalisateur » de l'apoptose, n'induit pas forcément à la mort. Ce phénomène introduisant l'activation de caspase-3 sous-létal et/ou locale est induit par la perméabilisation mitochondriale partielle. De manière intéressante, caspase-3 active localement joue des rôles non-apoptotiques dans les contextes physiologiques et pathologiques, par exemple, dans les réponses immunitaires, les cancers et le développement.

Cependant, les mécanismes régulant ce phénomène restent à découvrir. Les études ont récemment suggéré l'implication de la famille Bcl-2 dans ce processus.

La protéine Bcl-xL est une anti-apoptotique majeure de la famille. Sa surexpression a été observée dans plusieurs cancers, y compris le cancer du sein, de la prostate et le carcinome hépatocellulaire. Son rôle primordial dans la survie des neurones suggère son implication dans les pathologies neurodégénératives. Au cours du développement, Bcl-xL est essentiel. En effet, l'abrogation de *bcl-x*, le gène codant la protéine Bcl-xL, induit la mort embryonnaire, avec une augmentation de mort cellulaire dans les neurones et les cellules hématopoïétiques. Cependant, l'inhibition de Bcl-xL dans la culture de neurone ne mène pas directement à l'apoptose, mais produit les anomalies morphologiques, dont la croissance de neurites, la synaptogenèse ainsi que les malfonctionnements synaptiques.

Au plan cellulaire, Bcl-xL possède une double localisation subcellulaire : au niveau de la mitochondrie et du réticulum endoplasmique (RE). Sa localisation mitochondriale se lie directement à sa capacité d'inhiber les pro-apoptotiques et de prévenir la libération de cytochrome c. Au niveau du RE, Bcl-xL régule l'homéostasie calcique. Notre équipe s'est intéressé aux différences fonctionnelles de ces deux pools de protéine Bcl-xL. Nous avons ainsi généré deux modèles de souris exprimant Bcl-xL exclusivement soit au niveau du RE (souris ER-xL) soit à la mitochondrie (souris Mito-xL). Au contraire de nos attentes, l'absence de pool mitochondrial de Bcl-xL n'induit pas la mort embryonnaire chez les souris ER-xL. De plus, nous avons observé que l'activation de caspase-3 s'est présentée plus substantiellement dans la matière blanche que la matière grise de la moelle épinière et du cortex cérébrale de souris ER-xL. Les neurones corticaux dérivés des souris ER-xL présentent des signaux de caspase-3 active dans les neurites. Ces signaux, également trouvés dans les neurones WT, ont été amplifiés chez les neurones ER-xL. Cela a conduit à la perturbation du développement neuronal, y compris la diminution de l'arborisation axonale et l'abaissement de synapses formées et maturées. Nous avons pu observer également les changements au niveau de la mitochondrie suite à l'absence de la protéine Bcl-xL. En effet, les mitochondries dans l'axone des neurones ER-xL ont été fragmentées et possèdent un potentiel membranaire affaibli par rapport aux WT. Nous avons émis l'hypothèse que l'activation locale de caspase-3 pourrait impliquer une régulation de réseau mitochondrial. Par co-immunoprécipitation, nous avons montré que Bcl-xL, avec une séquence ciblant au RE, possède une interaction affaiblie avec la protéine Drp1, un régulateur de la fission mitochondriale, par rapport avec la Bcl-xL contenant une séquence transmembranaire WT. Ce résultat nous a suggéré que Bcl-xL régule négativement l'activité de

Drp1, ce qui a pour conséquence d'inhiber le découpage des mitochondries. En utilisant un inhibiteur de Drp1, nous avons établi le lien entre l'activation de caspase-3 axonale et l'augmentation de l'activité de Drp1 chez les neurones ER-xL.

En résumé, nos données nous ont permis de mettre en évidence un nouveau rôle non-canonique de la protéine Bcl-xL dans le développement neuronal. En régulant le réseau mitochondrial, Bcl-xL contrôle l'activation locale de caspase-3, ce qui est critique pour le développement normal des neurones.

Publications

Article :

Nguyen TTM, Gadet R, Lanfranchi M, Lahaye R, Yandiev S, Lohez O, Mikaelian I, Rimokh R, Courchet J, Saudou F, Pogeorgiev N, Gillet G.

Control of caspase-3 activity in the neurites by the mitochondrial pool of the apoptosis inhibitor Bcl-xl is critical for synaptogenesis in the developing nervous system

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Additional work:

1/ Popgeorgiev N, Sa JD, Jabbour L, **Nguyen TTM**, Akhavan-e-sabet A, Gadet R, Ralchev N, Manon S, Hinds MG, Osigus H, Schierwater B, Humbert PO, Rimokh R, Gillet G, Kavansakul M.

Ancient and conserved functional interplay between Bcl-2 family proteins in the mitochondrial pathway of apoptosis

Science Advance. September 30th, 2020.

Doi: 10.1126/sciadv.abc4149 (*Refer to ANNEX I*)

2/ Jabbour L, **Nguyen TTM**, Gadet R, Lohez O, Mikaelian I, Gonzalo P, Luyten T, Chalabi-Dcha M, Bultynck G, Rimokh R, Gillet G, Popgeorgiev N

The Endoplasmic Reticulum pool of Bcl-xL dampens the Unfolded Protein Response through IP₃R-dependent Calcium Release

BioRxiv. January 28th, 2021.

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Review article:

1/ **Nguyen TTM**, Popgeorgiev N, Gillet G. **Caspases in the Developing Central Nervous System: Apoptosis and Beyond**, *Frontiers in Cell and Developmental Biology*. July 16th, 2021. doi: 10.3389/fcell.2021.702404 (*Refer to ANNEX III*)

2/ Bas J, **Nguyen TTM**, Gillet G. **Involvement of Bcl-xL in Neuronal Function and Development**, *International journal of molecular sciences*. March 21th, 2001. Doi: 10.3390/ijms22063202 (*Refer to ANNEX IV*)

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”Rise above oneself and grasp the world”

Archimedes

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ABBREVIATIONS

+TIP: Plus-end tracking protein	cFLIP: Cellular FLICE/caspase-8-like inhibitory protein
AchR: Acetylcholine receptor	cIAP: Cellular inhibitor of apoptosis
AD: Alzheimer's disease	CNS: Central nervous system
ADF: Actin-depolymerizing factor (ADF)	CP: Capping protein
AIF-1: Allograft inflammatory factor 1	CUL9: Cullin 9
ALS: Amyotrophic lateral sclerosis	DARPP-32: Dopamine and cAMP-regulated phosphoprotein 32kDa
AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor	DCC: Deleted in colorectal cancer
AP-1: Activator protein 1	DCX: Doublecortin
Apaf-1: Apoptotic protease-activating factor 1	DD: Death domain
APC: Adenomatous polyposis coli protein	DED: Death effector domain
APP: β -amyloid precursor protein	DISC: Death-inducing signaling complex
Arp2/3: Actin-related protein 2/3	DKO: Double knockout
AS: Alternative splicing	Dnm2: Dynamin 2
ATP: Adenosine triphosphate	DRF: Diaphanous-related formin
AV: Active zone	DRG: Dorsal root ganglia
Bcl-2: B-cell lymphoma 2	Drp1: Dynamin-related protein 1
BDNF: Brain-derived neurotrophic factor	DUB: Deubiquitinase
BH: Bcl-2 homolog	Dvl1: Dishevelled segment polarity protein 1
BI-1: Bax inhibitor 1	EB3: End-binding protein 3
BSE: Bundle signaling element	ECFP: Enhanced cyan fluorescent protein
C3AI: caspase-3 activity indicator	ELK: ETS like-1 protein
Caf4: CCR4-associated factor 4	Ena/VASP: Enabled/vasodilator-stimulated phosphoprotein
CAM: Cell adhesion molecule	EndoG: Endonuclease G
CaMKII: Ca^{2+} /calmodulin-dependent kinase II	ER: Endoplasmic reticulum
CaN: Calcineurin	ERAD: Endoplasmic-reticulum-associated protein degradation
CARD: Caspase recruitment domain	ERK: Extracellular signal-regulated kinase
Cdc42: Cell division control protein 42	ETC: Electron transport chain
CDK: Cyclin-dependent kinase	

FA: Focal adhesion

F-actin: Actin filament

FADD: Fas-associated *via* death domain

FAK: Focal adhesion kinase

FasL: Fas ligand

FGF-8: Fibroblast growth factor 8

FRET: fluorescence resonance energy transfer

G-actin: Globular actin

GED: GTPase effector domain

GET: Guided entry of tail-anchored proteins

GGA3: Golgi-associated, gamma adaptin ear containing ARF binding protein 3

GRP75: Glucose-regulated protein 75

GSK-3: Glycogen synthase kinase 3

HBSS: Hank's buffered salt solution

HD: Huntington's disease

HDACi: Histone deacetylase inhibitor

HR1: Heptad repeat 1

HR2: Heptad repeat 2

HTT: Huntingtin

IAP: Inhibitor of apoptosis

IKK: I κ B kinase

iMOMP: Incomplete mitochondrial outer membrane permeabilization

IMS: Intermembrane space

INF2: Inverted formin 2

IP₃: inositol triphosphate

IP₃R: Inositol triphosphate receptor

JNK: Jun N-terminal kinase

KI: Knock-in

KIF2A: Kinesin family member 2A

KO: Knockout

LIMK: Lim kinase

LKB1: Liver kinase B1

LTD: Long-term depression

MAM: Mitochondrial-associated membrane

MAP: Microtubule-associated protein

MAPK: Mitogen-activated protein kinase

MAPL: Mitochondria-associated protein ligase

MCU: Mitochondrial Ca²⁺ uniporter

mDia2: Mouse homolog of Diaphanous 2

Mdv1: Mitochondrial division protein 1

MERC: Mitochondria-endoplasmic reticulum contact site

Mff: Mitochondrial fission factor

MICOS: mitochondrial contact site and cristae organizing system

MiD49: Mitochondrial dynamics protein 49

MiD51: Mitochondrial dynamics protein 51

MIM: Mitochondrial inner membrane

MKP-1: MAPK phosphatase-1

MMP: Matrix metalloproteinase

MOM: Mitochondrial outer membrane

MOMP: Mitochondrial outer membrane permeabilization

MPTP: Mitochondrial permeability transition pore

Mst1: Macrophage stimulating 1

Munc13: Mammalian uncoordinated 13

NCAM: Neural cell adhesion molecule

ND: Neurodegenerative disorder

NF- κ B: Nuclear factor kappa B

NGF: Nerve growth factor

NMDA: N-methyl-D-aspartate

NTC: Neural tube closure

NUAK1: Nuclear AMPK-related kinase 1

Opa1: Optic atrophy 1

OXPHOS: Oxidative phosphorylation

PARC: Parkin-like cytoplasmic protein

PCD: Programmed cell death

PD: Parkinson's disease

PH: Pleckstrin homology

PI3K: Phosphoinositide 3-kinase

PIDD: p53-induced protein with a death domain

PKA: Protein kinase A

PKB: Protein kinase B

PKC α : Protein kinase C α

PKC ζ : Protein kinase C ζ

PLK: Polo-like kinase

PNS: Peripheral nervous system

PP1: Protein phosphatase 1

PP2A: Protein phosphatase 2A

PSD95: Postsynaptic density 95

PTM: Posttranslational modification

Rac1: Ras-related C3 botulinum toxin substrate 1

Rb: Retinoblastoma protein

RGC: Retinal ganglion cells

RIM: Rab3-interacting molecule

RIM-BP: RIM binding protein

Robo: Roundabout

ROCK I: Rho-associated kinase I

ROS: Reactive oxygen species

SCAT3: sensor for activated caspase-3

Sema: Semaphorin

SERCA: Sarco/endoplasmic reticulum ATPase

SOCE: Store-operated Ca²⁺ entry

Spire1C: Spire type actin nucleation factor 1

SV: Synaptic vesicle

SVZ: Subventricular zone

TA: Tail-anchored protein

tBid: Truncated Bid

TGF β : Transforming growth factor β

TM: transmembrane

TNFR: Tumor necrosis factor receptor

TOM: Translocase of the outer membrane

TRAILR: Tumor-necrosis-factor-related apoptosis-inducing ligand

TRKA: Tropomyosin receptor kinase A

UPR: Unfolded protein response

USP9X: Ubiquitin-specific peptidase 9 X-linked

VDAC: Voltage-dependent anion channel

VZ: Ventricular zone

WAVE: Wiskott-Aldrich syndrome protein-family verprolin homologous protein 1

WT: Wild-type

XIAP: X-linked inhibitor of apoptosis protein

INTRODUCTION

1. Apoptosis, Bcl-2 family, and caspases

1.1. What is apoptosis?

Apoptosis, a word originating from ancient Greek, means “a falling off”, such as a leaf or a fruit from a tree when it is died or ripened. Kerr, Wylie, and Currie were the first to use it as a synonym for programmed cell death in 1972 (Kerr et al., 1972). Apoptosis, or programmed cell death (PCD) type I, is a controlled death of the unnecessary or damaged cells without affecting or harming neighboring cells (Norbury and Zhivotovsky, 2004).

For many years, cell death was divided into two types: regulated via apoptosis and unregulated via necrosis. With the discoveries of multiple regulatory pathways of necrosis, this point of view has changed: except for accident cases, cell death via necrosis is also highly regulated via necroptosis (Conrad et al., 2016). Apoptosis, autophagy, and necroptosis nowadays represent different types of programmed cell death (D’Arcy, 2019).

Apoptotic cells exhibit specific characteristics distinguishing them from necrosis, including cell shrinkage, chromatin condensation and fragmentation, and cell membrane blebbing, followed by apoptotic body formation (Taylor et al., 2008). This process maintains tissue homeostasis and sculpts morphology during the development of multicellular organisms (Elmore, 2007; Suzanne and Steller, 2013). Accordingly, apoptosis dysregulation is implicated in pathological conditions such as neurodegenerative disorders and participates in cancer progression (Friedlander, 2003; Cotter, 2009).

The mechanism of apoptosis is rather complex and involves a network of different molecules. In this cascade of events, the Bcl-2 protein family acts as a leading actor in sensing and inducing apoptosis. They control a central step in the apoptosis execution – the mitochondrial outer membrane permeabilization (MOMP). This key event leads to the release of cytotoxic molecules into the cytosol, which triggers the activation cascade of the executioners of apoptosis, the cysteine-aspartate proteases (caspase) family. Once activated, these proteases cleave a large subset of proteins and “escort” the cell toward its “final destination”, the death.

In the next parts, I will focus on these two protein families and their position in apoptosis.

1.2. Bcl-2 protein family – apoptosis regulator

1.2.1. History of the Bcl-2 family

B-cell lymphoma 2 (Bcl-2), the first identified member of the family, was discovered by its involvement in follicular lymphoma (Cleary et al., 1986). Indeed in the chromosomal translocation t(14;18) the *bcl2* gene is juxtaposed to immunoglobulin heavy chain gene enhancer elements, which leads to Bcl-2 overexpression (Tsujimoto et al., 1985; Vaux et al., 1988). The discovery of Bcl-2 protein brought to light a novel characteristic of cancer cells: their ability to silence apoptosis. Indeed, cancer cells not only possess an increased proliferation rate but also escape the apoptosis checkpoint, which further promotes the expansion of their population (Hanahan and Weinberg, 2000).

In 1993, three homologs of Bcl-2 were described: *mcl1* (encoding for Mcl-1) (Kozopas et al., 1993), *bcl2a1* (encoding for Bfl-1) (Lin et al., 1993), and *bcl2l1* (encoding for Bcl-xL and Bcl-xS) (Boise et al., 1993). All three proteins act as guardians against apoptotic death. In the same year, Bax was identified as a Bcl-2 binding partner. Bax: a Bcl-2 homolog triggers apoptosis rather than suppressing it as its relatives (Oltval et al., 1993). This work led Korsmeyer team to propose a widely accepted rheostat model of apoptosis regulation, according to which cell fate is determined by the ratio between the apoptosis repressor, Bcl-2, and the apoptosis accelerator, Bax.

Shortly after, a novel subset of the Bcl-2 family was identified, which groups only BH3 motif-bearing proteins. It includes Bik (Boyd et al., 1995), Bid (Wang et al., 1996), Bim (O'Connor et al., 1998), Bad (Yang et al., 1995), BNIP1, BNIP3 (Boyd et al., 1994), Noxa (Oda et al., 2000), Puma (Nakano and Vousden, 2001) (Figure 1). This subgroup introduced the third element into the rheostat model, which mediates cell death *via* antagonizing Bcl-2-like proteins and activating Bax-like proteins. The two-pole model became a “triad” model with more complexities. Indeed, BH3-only proteins interact with both anti-apoptotic and pro-apoptotic proteins and regulate their activities. They can directly activate Bax/Bak or inactivate Bcl-2-like proteins, therefore indirectly activating pro-apoptotic proteins. In contrast, the anti-apoptotic proteins also protect the cell against cell death *via* sequestering BH3-only proteins (Llambi et al., 2011).

Our knowledge about apoptosis regulation is founded on the work of Horvitz and his coworkers in the late 80s-90s using an invertebrate model. Their collection of work revealed that the apoptosis mechanism was highly conserved during evolution (Ellis and Horvitz, 1986; Yuan et

al., 1993; Hengartner and Horvitz, 1994). In *C. elegans*, apoptosis is executed by the caspase Ced-3. The caspase-activating Apaf-1 homolog Ced-4 performs the Ced-3 activation, which is inhibited by the Bcl-2-like protein Ced-9 in the non-stressful context. Upon cell death stimuli, the BH3-only protein Egl-1 is upregulated and recruits the Ced-9 protein, therefore liberating Ced-4 and activating apoptosis (Strasser and Vaux, 2018). Interestingly, human Bcl-2, when introduced into *C.elegans*, suppresses the cell death in this organism, suggesting the protein structure and interaction conservation between species (Vaux et al., 1992).

In vertebrates, Bcl-2 proteins are found to regulate the mitochondrial outer membrane permeabilization (MOMP), a key event of apoptosis induction that is absent in *C.elegans* (Kalkavan and Green, 2018). Upon MOMP, Apaf-1 is activated through cytochrome c binding and ultimately leading to executor caspase activation, similar to the invertebrate model.

Since their discovery, the Bcl-2 family of proteins has been actively studied for their substantial implication in human pathologies (Pentimalli, 2018).

In cancer, Bcl-2-like proteins are often overexpressed to overcome apoptosis. Thus, they represent potential targets in the cancer context. Among the efforts to re-establish cell death, the most successful is targeting Bcl-2-like proteins *via* molecules mimicking the BH3 motif of BH-only proteins known as “BH3 mimetics” (Delbridge and Strasser, 2015). These molecules block the anti-apoptotic activity of Bcl-2 proteins, thus re-establishing apoptosis.

In contrast to cancer, neurodegenerative diseases are induced by excessive apoptosis in neurons. For example, neuron cell death in the hippocampus and cerebral cortex results in Alzheimer's disease; apoptosis of dopaminergic neurons in the midbrain causes Parkinson's syndromes; the apoptosis of striatal neurons correlates with Huntington's disease. In these pathologies, a large body of evidence hints implication of Bcl-2 family members in the neurodegenerative pathologies (Sassone et al., 2013). However, targeting Bcl-2 proteins in these diseases is still in its beginning.

Interactions between Bcl-2 proteins were the center of study for decades since they were the main factor defining the cell fate (Kale et al., 2018b). Aside from mitochondria – the first described site governing these interactions, emerging evidence of other interaction sites has been reported. In addition, recent studies highlight that Bcl-2 proteins, besides interaction among themselves, have a vast interactome with diverse cellular pathways (Popgeorgiev et al., 2018). In one way, this suggests that Bcl-2 proteins function as an intersection point linking complex networks of cellular pathways to make ultimate cell fate decisions. In other words, it

potentially means that besides cell death regulation, Bcl-2 proteins play non-apoptotic roles in physiological contexts involving metabolism, signalization, and development (Bonneau et al., 2013; Glab et al., 2020).

Here, we present the timeline of main events in Bcl-2 protein identification and Bcl-2-related therapeutics (Figure 1).

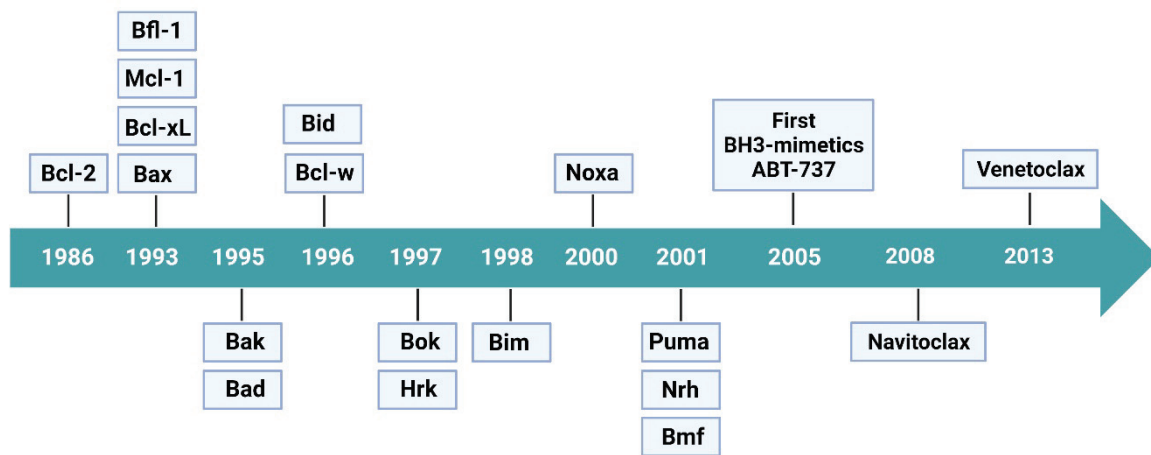


Figure 1. Timeline of discoveries from mammalian Bcl-2 proteins to Bcl-2-related therapeutic development.

The cloning year of sixteen mammalian Bcl-2 proteins and BH3 mimetics (navitoclax and venetoclax) development are indicated.

1.2.2. Bcl-2 family classification and structure

1.2.2.1. Classification

Since the discovery of the first protein, the Bcl-2 family has expanded dramatically. Genes coding for Bcl-2 proteins have been found in almost all metazoans suggesting that these proteins are closely associated with the multicellular character of the animal kingdom (Kvansakul and Hinds, 2015; Popgeorgiev et al., 2020). Hitherto, seventeen proteins have been identified in mammals.

In order to be classified as a Bcl-2 member, one protein needs to contain at least one evolutionarily conserved amino acid sequence, termed the Bcl-2 homology (BH) motif (Kvansakul and Hinds, 2015). Four conserved motifs have been identified, referred to as BH1-4 motifs. BH1 and 2 are the most conserved BH motifs, whereas the BH4 is the most divergent. Moreover, the presence of the BH3 domain is obligatory for the pro-apoptotic effect. Most Bcl-2 protein members also possess a C-terminal transmembrane (TM) helix, which is necessary to determine the cellular localization of the host protein (Petros et al., 2004).

Based on their structure and their activity to induce or block apoptosis, Bcl-2 proteins are classified into three subgroups: multi-domain anti-apoptotic, multi-domain pro-apoptotic, and BH3-only proteins. In mammals, there are six multi-domain anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1, and Nrh), three multi-domain pro-apoptotic (Bax, Bak, and Bok), and seven canonical BH3-only proteins: Bik, Bim, Bad, Bmf, Noxa, Puma, Hrk (Youle and Strasser, 2008; Kvansakul and Hinds, 2015) (Figure 2).

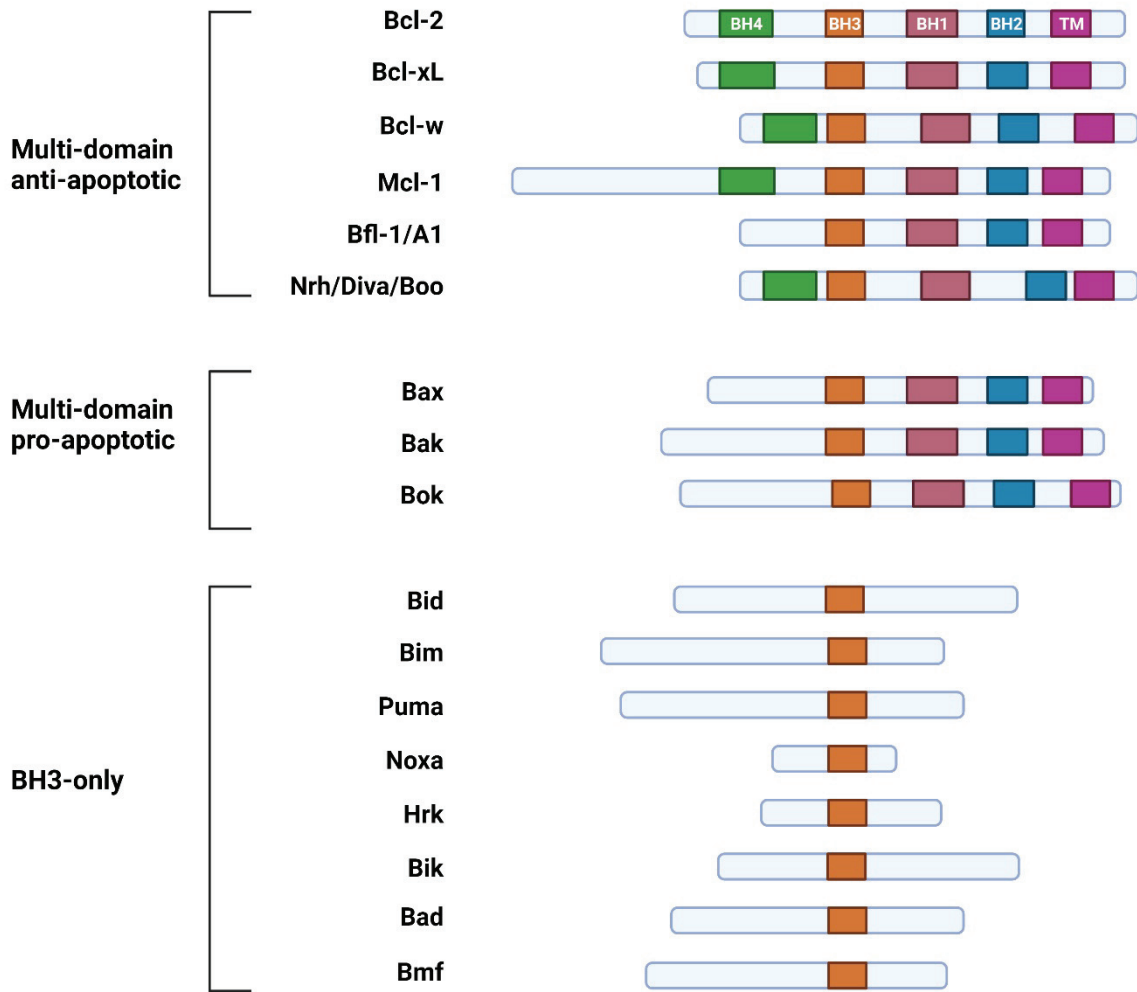


Figure 2. Classification of mammalian Bcl-2 proteins.

Conserved BH and TM motifs of these Bcl-2 family proteins are indicated. Protein length is roughly scaled.

1.2.2.2. Protein structure

Multi-domain anti-apoptotic proteins

Multi-domain anti-apoptotic proteins are the only members of the Bcl-2 family that bear all four BH domains. They are organized into eight α -helix folded to a highly conserved 3D structure (Petros et al., 2004).

Bcl-2 anti-apoptotic proteins possess a structurally conserved hydrophobic groove constituted by BH1-3 domains on their surface. This structure forms a liaison with the amphiphilic α -helix segment of the BH3 motif of multi-domain pro-apoptotic protein and BH3-only protein (Sattler et al., 1997). Even though the anti-apoptotic hydrophobic groove structure is highly similar between Bcl-2 members, they display undeniable diversity of specificity. Indeed, they have different preferences for BH3-only protein. For instance, Mcl-1 binds to Noxa but not to Bad which is different from the Bcl-2, Bcl-xL, and Bcl-w binding repertoire (Kale et al., 2018b). The specificity of this groove may come from the difference in primary structure, regardless of their similitude in tertiary structure. The interaction between the BH3 motif of pro-apoptotic proteins and Bcl-2 anti-apoptotic proteins is known as the canonical interaction of the Bcl-2 protein family. This interaction is the basis for BH3-mimetics development as a cancer therapy. A recent study showed that the BH4 motif of Bcl-2 binds to Bax in a new site located in a groove formed by residues of α 1, α 1– α 2 loop, and α 2– α 3 and α 5– α 6 hairpins on the BAX surface. This non-canonical interaction constrains Bax conformational changes during its activation (Barclay et al., 2015).

Actually, the BH4 is a 20 amino acids long motif located in the N-terminus part of the Bcl-2 proteins. It has the lowest sequence similarity of all BH motifs and organizes into an α -helix (Monaco et al., 2012). Notably, BH4 is essential for the anti-apoptotic effect of Bcl-2-related proteins. Indeed, when this sequence is dampened by alternative splicing (AS) or eliminated by proteolytic cleavage, the host protein loses its anti-apoptotic function and acquires pro-apoptotic properties (Hanada et al., 1995; Cheng et al., 1997; Huang et al., 1998).

Moreover, BH4 is likely to be a platform for interaction with multiple non-Bcl-2-related proteins, including Inositol triphosphate receptor (IP₃R), Voltage-dependent anion channel (VDAC), and Ras (Liu et al., 2016). These interactions may participate in the control of apoptosis as well as in non-apoptosis-related processes (see part 2), which makes interesting strategies that target the BH4 sequence prominent in cancer therapy (Nougarède et al., 2018).

Besides the BH motifs, Bcl-2 anti-apoptotic proteins also contain a hydrophobic TM sequence at their C-terminus. This motif, composed of approximately 20 amino acids, is folded into an α -helical structure (Kvansakul and Hinds, 2015). TM domain is essential for protein targeting and localization, which is essential for protein function (Popgeorgiev et al., 2018).

Multi-domain pro-apoptotic proteins

Multi-domain pro-apoptotic proteins, Bax and Bak, were usually described with three BH domains, the BH1-3. A later study suggests a novel consensus sequence motif for the BH4 domain, which both core anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1) and pro-apoptotic (Bax, Bak) possess (Kvansakul et al., 2008).

The 3D structure of Bax and Bak exhibits a high similitude with Bcl-2 and Bcl-xL despite their low percentage identity in the amino acid sequences (Figure 3). Both Bax and Bak consist of nine α -helices, including the helical fold of the TM domain. The amphipathic helices surround the central hydrophobic α 5 helix. The BH1-BH3 domains fold into a hydrophobic pocket. In the resting state, the α 9 helix of Bax, which corresponds to its TM domain, binds to its proper hydrophobic pocket (Westphal et al., 2014b). On the one hand, it renders the hydrophobic pocket inaccessible; on the other hand, it buries the membrane-targeting sequence. Consequently, under the inactive form, Bax stays in the cytosol.

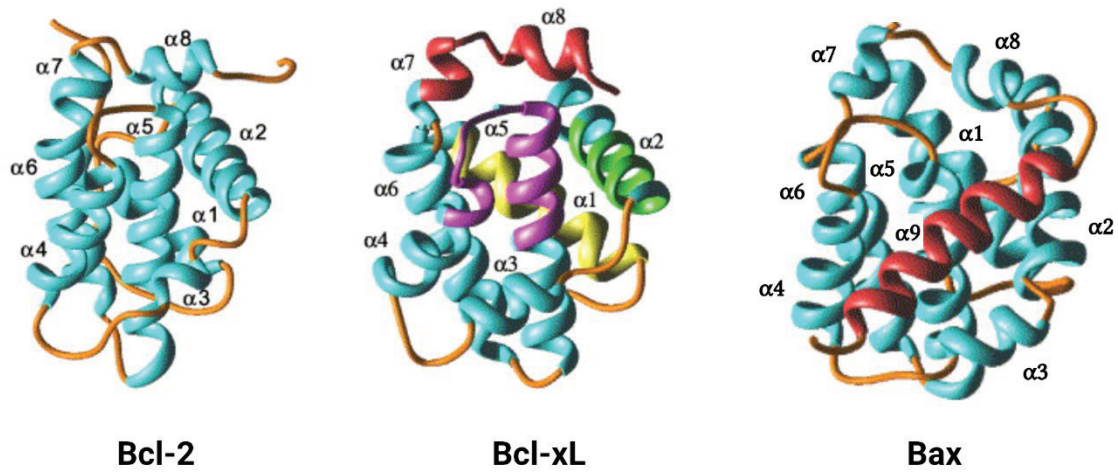


Figure 3. Ribbon representative of Bcl2, Bcl-xL, and Bax protein.

Helices are numbered from the N-terminus of protein. For Bcl-xL, the magenta ribbon represents the BH1 domain, the red represents the BH2, the green represents the BH3, and the yellow represents the BH4. The TMD of Bcl-2 and Bcl-xL is not included. For Bax, the red ribbon represents the TMD. Adapted from (Petros et al., 2004).

Regarding Bak, the hydrophobic pocket is narrowed down by its side chain, possibly hampers $\alpha 9$ recruitment (Petros et al., 2004). Moreover, $\alpha 9$ of Bak is more hydrophobic than Bax, which may explain its preference for the membrane's hydrophobic environment rather amphipathic environment of the groove (Westphal et al., 2014b).

At the resting state, the peptide sequence, which mediates the interaction between the BH3 domain of Bax and anti-apoptotic proteins, is directed toward the hydrophobic groove and is further covered by its $\alpha 9$ helix (Westphal et al., 2011). In order to expose this sequence to interact with anti-apoptotic proteins, a conformational change, in which $\alpha 9$ is removed from the groove and $\alpha 2$ rotates to reveal these components, is necessary (Westphal et al., 2014b). Such modifications occur when Bax is addressed to the mitochondrial outer membrane (MOM). Edlich and colleagues proposed that Bax translocates continuously to the MOM in healthy cells and is constantly redirected back to the cytosol by Bcl-xL (Edlich et al., 2011). However, the mechanism of continuous Bax shuttling to the MOM is elusive.

Despite its identification over twenty years ago, our knowledge about Bok functions is far from complete. First discovered in rat ovaries, testis, and uterus, Bok showed its “killer” functions under overexpression conditions (Hsu et al., 1997). With its homolog sequence to Bax and Bak, Bok is classified in the same group as these two pro-apoptotic proteins. A recent structural study confirmed the similar 3D structure between these three proteins (Ke et al., 2018; Zheng et al., 2018).

BH3-only proteins

BH3 motif was first identified in the Bak protein and then recognized in Bik (Chittenden et al., 1995). This discovery founded a novel class of Bcl-2 protein, termed BH3-only protein. Compared to others, the unique feature of this class of protein is that they contain only one conserved BH motif - the BH3 motif, which is designed for pro-apoptotic propriety of Bcl-2 proteins as it can be absent in some anti-apoptotic members.

Since the identification of Bid, the number of BH3-only proteins has increased extensively. For now, eight core BH3-only proteins are detected in mammals: Bik, Bim, Bad, Bmf, Noxa, Puma, and Hrk. The BH3-like motif can be found in a diversity of proteins; even some are not recognized as Bcl-2 proteins (Aouacheria et al., 2013). From a strict point of view, a protein can be classified as a BH3-only protein only if it bears a BH3-like motif with conserved residues derived from known BH3-only protein and binds with reasonable interaction to Bcl-2 anti-apoptotic protein and is demonstrated for a direct role in apoptosis induction (Kvansakul and

Hinds, 2015). For instance, based on these criteria, Beclin-1 is not a BH3-only protein. However, this notion is rather evolving and may be adapted with future research studies.

Based on structural analysis and sequence study, a 13-residue motif has been proposed as the core of the BH3 motif (Day et al., 2008; Lomonosova and Chinnadurai, 2008). Leucine and aspartic acid residues are the most conserved and thought to be necessary for BH3 motif interaction (Kvansakul and Hinds, 2014; Aouacheria et al., 2015).

Unless Bid presents a well-defined structure, the other BH3-only proteins are intrinsically disordered. They only adopt a structured BH3 region after binding to their multi-motif Bcl-2 partners. Structural studies showed that BH3-only proteins fold into an α -helix when binding to their Bcl-2 partners. Due to their unstructured nature, in comparison to completely folded Bcl-2 multi-motif, it is thought that two classes of protein are phylogenetically separated (Hardwick et al., 2012).

Following the divergence in the sequence, BH3-only proteins exhibit different specificity with their partners. There are two clear tendencies in this subgroup in order to induce apoptosis: *via* direct interaction with pro-apoptotic Bax/Bak (Bim, Puma, Bid) and *via* inhibiting the anti-apoptotic proteins (Bad, Bik, Noxa) (Kale et al., 2018b).

1.2.3. Cellular localization of Bcl-2 proteins

1.2.3.1. Mechanism of Bcl-2 protein docking

In order to execute their functions, Bcl-2 proteins must be directed to their target intracellular membrane. The mechanisms mediating the localization of these proteins remained to elucidate better. Several Bcl-2 proteins, like Bak, Bcl-2, and Bcl-xL, are distributed directly on their sites of action while the others, including Bax, Bcl-w, and several BH3-only proteins, translocate only to their target sites in response to apoptotic stimuli (Lindsay et al., 2011). All multi-motif Bcl-2 proteins possess a TM motif at the C-terminus. Therefore, they are classified into the group of tailed-anchor proteins. Like other tailed-anchor proteins, the TM motif of Bcl-2 proteins is organized into α helices (Lindsay et al., 2011). Information for the membrane insertion is likely encoded on the TM motif. Indeed, Bcl-2 without its TM motif does not address its frequent membrane localization but stays cytosolic (Nguyen et al., 1993). Moreover, the TM motif is sufficient to drive GFP to the target membrane.

It is worth noting that the TM motif of Bcl-2 proteins, with high hydrophobicity, is synthesized lastly when the N-terminal part is already folded. Therefore, to prevent aggregation, protein chaperons or cytosolic factors are expected to bind and hide these hydrophobic structures before

protein insertion into its target membrane (Beddoe and Lithgow, 2002; Schinzel et al., 2004). However, up-to-date, there are no such elements documented in the literature.

The TM motif also bears the information for the membrane specificity of Bcl-2 proteins, which drives relative proteins to different final destinations. For instance, Bcl-2 and Bcl-xL possess TM motifs with a similar level of hydrophobicity. Nonetheless, Bcl-xL locates principally at the mitochondria and a minority at the ER and cytosol, whereas Bcl-2 addresses the ER, mitochondria, and nuclear envelope (Krajewski et al., 1993; Kaufmann et al., 2003). The preference toward the MOM rather than ER is due to the residues flanking the TM motif. Indeed, Kaufmann and colleagues found that the TM motif of Bcl-xL situates between two positively charged amino acids, which are absent in Bcl-2. When their TM motifs are exchanged, their localization is inverse (Kaufmann et al., 2003).

Even the study of Kaufmann and colleagues explains why Bcl-xL is preferentially at MOM while Bcl-2 is distributed equally between ER and MOM, it does not clarify how these two proteins, in particular, and other Bcl-2 proteins in general, can be found in multiple intracellular membranes. For instance, even though Bcl-xL possesses a “bonafide” MOM targeting sequence, it can also be found in the ER (White et al., 2005; Monaco et al., 2012). This observation suggests that the topogenic sequence is insufficient to direct the host protein toward a target route. Additional information, for instance, binding proteins, interlinks with the TM motif to direct proteins to their final destination, in accordance with the cellular context.

Another question remaining to be answered is whether active transport mechanisms, such as a protein shuttle system or sorting receptors, involve in the targeting process of Bcl-2 proteins. In a study realized on yeast, the TM motif of Bcl-2 protein was found to bind to TOM20. TOM20 is a member of the Translocase of the outer membrane (TOM) family. It is highlighted as a receptor recognizing mitochondria-targeted protein and enhancing the membrane insertion of TA proteins (Endo and Kohda, 2002; Horie et al., 2003; Doan et al., 2020). Guided Entry of Tail-anchored protein (GET) complex is also demonstrated to involve in ER membrane insertion of TA proteins (Schuldiner et al., 2008). These studies hint the putative role of different protein complexes in targeting TA proteins in general and Bcl-2 proteins in particular.

When a TA protein has multiple target organelles, as in the case of multiple Bcl-2 proteins, a change in one targeting pathway may lead to more protein distribution to other pathways. For instance, when the mitochondria protein import system is compromised or overwhelmed, the GET complex may interfere and direct mitochondrial protein toward ER (Vitali et al., 2018).

The implication of different sorting and transport pathway also gives a platform to re-allocate the ratio of one protein on different cellular organelles *via* directing newly synthesized molecules. As well, it is interesting to ask if a mechanism to redirect already-inserting protein into another organelle membrane in response to a contextual stimulus.

1.2.3.2. Bcl-2 protein action sites

As mentioned above, most Bcl-2 proteins are found on multiple intracellular sites. Since the first documented site – mitochondria, a larger setting has been described now: from ER, nucleus, to Golgi apparatus, lysosome, and peroxisomes (Popgeorgiev et al., 2018). At these sites, Bcl-2 proteins are thought to control in a remote way apoptotic events at MOM.

Most Bcl-2 family members are attached to the MOM: some are addressed to the MOM even at the “healthy state” like Bak, Bcl-xL; some only translocate to the MOM upon apoptotic stimulus. For instance, Bid needs to undergo proteolytic cleavage to permit its translocation (Li et al., 1998). Other BH3-only proteins like Bad and Bim only move to the MOM when their binding partners are neutralized or dissociated (Zha et al., 1996; Puthalakath et al., 1999). Multiple Bcl-2 anti-apoptotic proteins were also found in other compartments of mitochondria. Bcl-xL is detected at the mitochondrial inner membrane (MIM), while Mcl-1 also localizes in the matrix. This pool of Bcl-2-like proteins is likely to promote mitochondrial bioenergetics (Alavian et al., 2011; Perciavalle et al., 2012).

Another main localization of Bcl-2 proteins is the ER. Indeed, both anti- and pro-apoptotic proteins, including multi-motif and BH3 only, reside on the membrane of this organelle (Scorrano et al., 2003; Zong et al., 2003; White et al., 2005; Heath-Engel et al., 2012).

The ER, a network of membranes expanding from the nucleus over the cell, has critical functions in the cell’s life: it operates as a center of protein folding, lipid synthesis, and cellular calcium storage. Thus the ER has a central role in the cellular homeostasis and involves a complex cross-talk between the unfolded protein response, calcium signaling, and mitochondrial bioenergetics (Schwarz and Blower, 2016). Under stress, ER may engage in a condition called the unfolded protein response (UPR). The UPR aims to create an adaptive program to rescue the ER protein folding capacity. If the stress endures or ER homeostasis cannot be re-established, the UPR can trigger apoptosis to eliminate the damaged cell (Hetz, 2012). Bcl-2 proteins, especially BH3-only, function as the molecular linker between UPR and apoptosis mitochondrial pathway. For instance, BOK locates mainly in the ER and is continually eliminated *via* the ER-associated degradation (ERAD) pathways (Llambi et al.,

2016). When ERAD is affected under the UPR, BOK is stabilized and translocated to the MOM and triggers apoptosis (Carpio et al., 2015; Llambi et al., 2016).

Bcl-2 proteins are also implicated in calcium homeostasis in the ER. They interact with the inositol triphosphate receptor (IP₃R), a calcium release channel. The IP₃Rs are abundant in the ER specialized subdomain, mitochondria ER-associated membranes (MERC), an inter-organelle platform promoting calcium transfer, lipid transfer, mitochondria metabolism, and apoptosis (Bartok et al., 2019). An overload of mitochondria calcium leads to Bax/Bak independent cytochrome c release *via* a little-known structure named mitochondrial permeability transition pore (MPTP) (Szalai et al., 1999). Thus, mitochondria-related cell death can be triggered by ER *via* prolonged calcium ER-mitochondria flux. Such signals can be inhibited by anti-apoptotic proteins, including Bcl-2 and Bcl-xL (Rong et al., 2009; Rosa et al., 2022). Bcl-2, *via* its BH4 motif, interacts with the regulatory and coupling domain of IP₃R and keeps the calcium channel at the closing state (Rong et al., 2009).

Later, work from Foskett's lab showed that anti-apoptotic proteins might interact with other regions of IP₃R. At low concentrations of IP₃ in the physiological context, they bind to the C-terminal of IP₃R and promote small calcium oscillation to enhance mitochondrial bioenergetics (White et al., 2005). This phenomenon was first described in the Bcl-xL, then later observed in two other anti-apoptotic proteins, Bcl-2 and Mcl-1 (White et al., 2005; Yang et al., 2016). This work suggests that anti-apoptotic proteins promote cell fate toward life direction *via* different interactions to IP₃R dependent cellular contexts (White et al., 2005; Eckenrode et al., 2010, 1; Yang et al., 2016). However, recent work from the Bultynck lab shows discordances with the model proposed by Foskett work. In the latter work, Bultynck and his colleagues reported the similarities of Bcl-xL and Bcl-2 in the interaction with IP₃R. Even at a low concentration of IP₃, they did not observe the IP₃R sensitizing effect of Bcl-xL as reported in the previous work (Rosa et al., 2022).

Bax and Bak are also located at the ER (Scorrano et al., 2003; Zong et al., 2003). Even though direct interaction between Bax/Bak and IP₃R has not been detected, these two proteins are documented to promote ER-mitochondria calcium transfer. Indeed, ER calcium release is altered in Bax-null cells and is re-established when Bax is re-introduced (Nutt et al., 2002a). In addition, overexpression of Bax/Bak leads to ER calcium depletion concurrently with increased mitochondria calcium uptake (Nutt et al., 2002b). In this respect, Bcl-2 proteins interact with VDAC at the mitochondria and regulate this mitochondrial calcium uptake channel (Shimizu et al., 1999; Monaco et al., 2015).

Besides, some Bcl-2 proteins are found in other cellular compartments, including the nucleus, peroxisome, lysosomes, and Golgi body (Popgeorgiev et al., 2018). That opens new sight for future studies to further knowledge about the non-canonical roles of the Bcl-2 protein family.

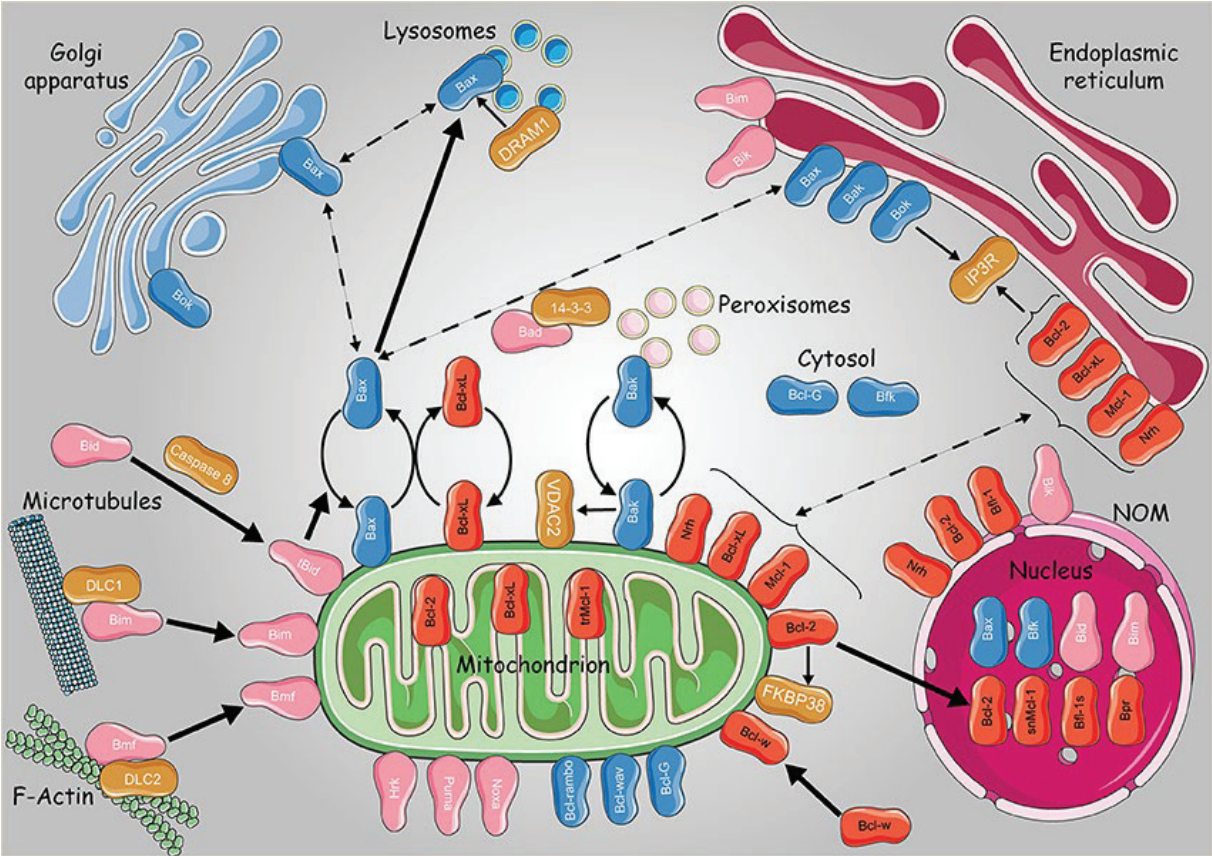


Figure 4. Subcellular dynamics of Bcl-2 proteins

The red blocks represent anti-apoptotic proteins, the blue ones represent multi-apoptotic proteins, and the pink ones represent BH3-only proteins. The brown blocks represent non-Bcl-2 proteins. The solid arrow indicates documented protein translocation from one organelle to another. The dashed lines illustrate hypothetical translocation (Popgeorgiev et al., 2018).

1.2.4. Interaction between Bcl-2 proteins

1.2.4.1. Basis of Bcl-2 interaction

The multi-motif members, both anti- and pro-apoptotic proteins, contain a hydrophobic pocket formed by $\alpha 2$ - $\alpha 5$ helices (Petros et al., 2004). This hydrophobic groove can interact with the BH3 motif of BH3-only proteins, which forms the ground of the interplay between Bcl-2 family proteins (Shamas-Din et al., 2013). The interaction between Bcl-2 proteins dictates the destination of the cell towards apoptosis or healthy life.

In a simple “rheostat model”, it is proposed that the ratio between anti- and pro-apoptotic proteins defines the apoptotic fate of a cell. This model was conceived based on the sole interaction between Bax-like and Bcl-2-like members. With a more expanding Bcl-2 family, especially with BH3-only protein discovery, this is reformulated: cell fate is defined by the sum of interactions between anti- and pro-apoptotic proteins (Llambi et al., 2011).

BH3-only proteins are divided into activators and sensitizers. When the BH3 motif of an activator binds to the hydrophobic groove of Bax/Bak, it activates these two pro-apoptotic proteins. Conversely, sensitizers promote apoptosis *via* inhibiting the anti-apoptotic proteins, such as Bcl-2, Bcl-xL, and Mcl-1 (Shamas-Din et al., 2011). Biochemical data from peptide-based and full-length protein interaction reveal that almost BH3-only proteins are capable of binding to both Bax-like and Bcl-2-like members with different affinity (K_D) (Table 1) (Kale et al., 2018b). That means a BH3-only is classified based on its preferred binding partners.

In the intracellular environment, the affinity between two partners is under the regulation of post-translational modifications and the abundance of other Bcl-2 and no – Bcl-2 partners (Kale et al., 2018b).

	Bim	Bid	Puma	Noxa	Bmf	Bad	Hrk	Bik
Bcl-2	Dark Green	Light Green	Dark Green	Yellow	Dark Green	Dark Green	White	Yellow
Bcl-Xl	Dark Green	Dark Green	Dark Green	Yellow	Dark Green	Dark Green	Dark Green	Dark Green
Bcl-w	Dark Green	Dark Green	Dark Green	White	Dark Green	Light Green	Dark Green	Dark Green
Mcl-1	Dark Green	Dark Green	Dark Green	Dark Green	Yellow	White	Yellow	Yellow
Bfl-1	Dark Green	Dark Green	Dark Green	Dark Green	White	White	Light Green	Dark Green
Nrh	White	White	White	White	White	White	White	White
Bax	White	Dark Green	White	White	White	White	White	White
Bak	Light Green	Yellow	Light Green	Yellow	White	White	White	White

Table 1. Bcl-2 protein family member interactions between the multi-motif proteins and BH3-only protein

The dissociation constant (K_D) values of the corresponding interaction are indicated on a color scale: the dark green represents a $K_D < 50\text{nM}$, the light green represents a K_D of 50-300nM, and the yellow represents a K_D of 300-10000nM. Adapted from (Kale et al., 2018b)

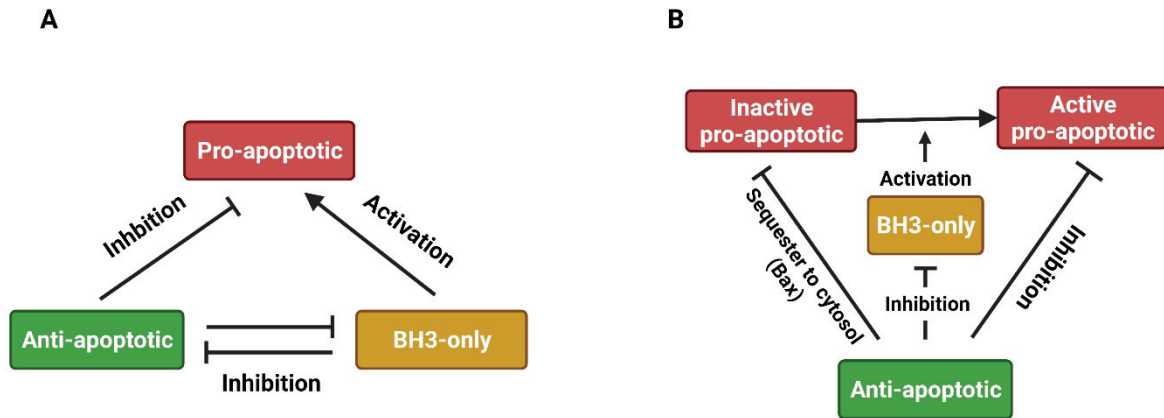


Figure 5. Schema of Bcl-2 interaction network

(A) Interactions between three subgroups of Bcl-2 proteins. The anti-apoptotic proteins (in green) inhibit the activities of the pro-apoptotic and BH3-only proteins. In contrast, the BH3-only proteins can activate apoptosis by activating pro-apoptotic proteins (direct way) or inhibiting the anti-apoptotic proteins (indirect way). **(B)** Three modes of pro-apoptotic inhibition by anti-apoptotic proteins. The anti-apoptotic proteins can sequester the inactive form of the pro-apoptotic protein at the mitochondria (exclusive for Bax). They also inhibit directly active forms of the pro-apoptotic proteins or inhibit BH3-only induced Bax/Bak activation. Adapted from (Westphal et al., 2014b)

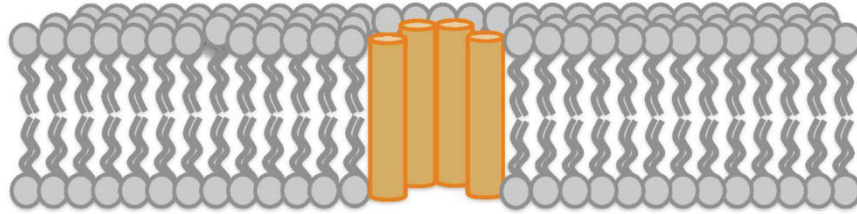
1.2.4.2. Activation of Bax and Bak – pore formation

The activation of Bax and Bak is a multi-step process of conformation changes leading to a pore-like structure formation by oligomerization on the MOM. In the inactive state, Bax and Bak preserve a slight difference: the hydrophobic TM motif on the $\alpha 9$ helix of Bax is sequestered into the hydrophobic groove of protein (Suzuki et al., 2000). Therefore, inactive Bax is cytosolic, whereas Bak locates at MOM even at the resting state. When binding to an activator, the $\alpha 9$ helix is displaced from the protein core, then is targeted on the MOM, letting Bax adopt the similar configuration as Bak. From this step, Bax and Bak activation shares high similarities (Westphal et al., 2011).

Furthermore, part of $\alpha 5$ and $\alpha 6$ are partially inserted into MOM with $\alpha 9$, leading to a more stable transmembrane configuration (Bleicken et al., 2014; Westphal et al., 2014a). Additionally, conformation changes then lead to the exposition of the hydrophobic groove by expelling the $\alpha 1$ and $\alpha 6-8$ regions out of the pocket (Westphal et al., 2014b). When changing into active transmembrane monomer conformation, Bax and Bak were thought to lose their high affinity with the BH3-only activator that then is sequestered by other inactive monomers. The release of BH3 only from the hydrophobic pocket of active Bax and Bak allows homodimer formation *via* interaction between the BH3 motif of the binding groove (Eskes et al., 2000; Wei et al., 2000). Dimer units of active Bax/Bak are then rearranged rapidly into a large ring-like pore. This structure is stabilized by intramembrane interaction between $\alpha 9$ helix (Zhang and Armstrong, 2007).

The nature of Bax/Bak pore remains obscure. Two proposed models are proteinaceous or a mixed lipid-protein pore (Figure 6) (Westphal et al., 2011). Data from Bax pore formation in the liposome hitherto favor the hypothesis of a mixed model (Terrones et al., 2004; Qian et al., 2008)

A. Proteinaceous pore



B. Toroidal pore

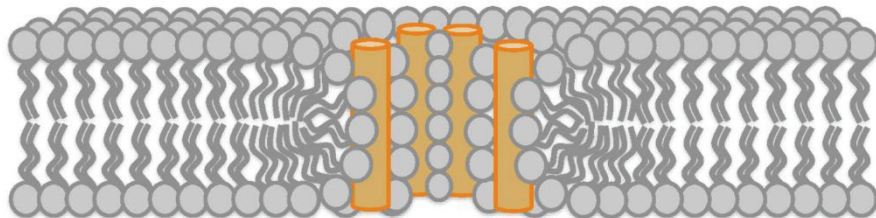


Figure 6. Two proposed models for Bax/Bak pore formation at the MOM

In model A, helical structures align to form a proteinaceous barrel. In model B, helical peptides create a curvature in the lipid membrane and form a pore mixing of protein and lipid heads (Westphal et al., 2011).

1.2.4.3. Inhibition of Bax and Bak activation by anti-apoptotic proteins

Via interaction with BH3-only activators

The Bcl-2 anti-apoptotic proteins indirectly inhibit Bax/Bak activation by sequestering the BH3-only activators (Kim et al., 2006b). This interaction involves the BH3 motif of BH3-only and the hydrophobic pocket of the anti-apoptotic protein.

Via direct inhibition of active Bax and Bak

Anti-apoptotic proteins might inhibit pore formation of Bax and Bak *via* direct interaction with active Bax/Bak monomer (Leber et al., 2010). The exposed BH3 motif of Bax/Bak is recruited to the hydrophobic groove of Bcl2-like proteins. This interaction prevents the homodimerization between Bax/Bak and impedes pore formation.

To execute this function, Bcl-2 anti-apoptotic proteins first undergo conformation changes to expose their hydrophobic pocket. That is likely induced when an anti-apoptotic bind to BH3-only protein, as proposed in the case of Bax/Bak. In the case of Bcl-2 protein, interaction with BH3-only induces the engagement of $\alpha 5$ and $\alpha 6$ into the MOM, which is essential to heterodimer formation with Bax/Bak (Bleicken et al., 2014). Otherwise, when binding to BH3-only, Bcl-w displaces its TMD, which permits tight insertion into the MOM and exposure of the hydrophobic grooves covered by the TMD in the inactive form (Hinds et al., 2003).

Thus, in the presence of the apoptotic stimuli, the anti-apoptotic proteins act concurrently as a negative competitor for BH3-only protein and direct inhibitor to Bax/Bak.

Via retro-translocation of Bax

In healthy cells, Bax is maintained in the cytosol due to an active system of retro-translocation. Indeed, Bax is in a continuity of mitochondria targeting and cytosolic redirecting. Edlich et al. have proposed that Bcl-xL facilitates Bax retro-translocation. According to this model, Bcl-xL binds to Bax on the MOM. This duo then travels together towards the cytosol and then separates. The interaction between Bax and Bcl-xL is *via* the BH3 motif of Bax (Edlich et al., 2011). That means before interacting with Bcl-xL, Bax has to undergo certain changes already to expose its BH3 motif. Thus, it is hypothesized that Bcl-xL redirects pre-activated Bax toward cytosol and protects the integrity of MOM.

Even Bax retro-translocation is described in the case of Bcl-xL protein, Bcl-2 and Mcl-1 are likely to share this feature as overexpression of these two Bcl-2 proteins increases the retro-translocation rate of Bax (Edlich et al., 2011). However, as Bcl-2 and Mcl-1 have not been

found or been found in very small quantities in the cytosol (Krajewski et al., 1993; Zhong et al., 2005), it is thought that Bax retro-translocation is mainly assured by Bcl-xL.

1.2.4.4. Activation of Bax/Bak by BH3-only protein

Bax and Bak are kept in check in healthy cells by anti-apoptotic protein *via* direct and indirect inhibition. When presents an apoptotic stimulus, antagonizing capacity of anti-apoptotic proteins might be overcome by BH3-only protein upregulation. An increase of BH3-only sensitizers blocks Bcl-2-like proteins and releases Bax/Bak and BH3-only activators. In turn, BH3-only activators activate Bax/Bak, which then trigger pore formation at MOM (Kim et al., 2006b; Leber et al., 2010).

Altogether, cell fate is determined by a competitive interaction triad between three subgroups of Bcl-2 proteins, which composes a confining system to regulate apoptosis.

1.2.5. MOMP: central regulation of Bcl-2 proteins

Bcl-2 proteins are major regulators of MOM integrity. When MOM becomes permeabilized in a process termed MOM permeabilization (MOMP), intermembrane space proteins are released into the cytosol and initiate, in most cases, a lethal apoptosis cascade. Thus, MOMP represents a highly regulated checkpoint process (Kalkavan and Green, 2018).

1.2.5.1. Characteristics of MOM and MIM

Mitochondrion, a semi-autonomous organelle, is isolated from the cytosol by double membranes: the MOM and MIM. The MOM is a less selective barrier than the MIM. Indeed, ions and small molecules pass freely through pore-forming membrane proteins (porins). Large molecules, including proteins, are imported through special transporters known as translocases. In contrast, the transport through MIM is much more regulated as this membrane is impermeable (O'Rourke, 2007). Molecules, including ions and macromolecules, only cross with specific membrane transport proteins, including uniporters, antiporters, and symporters (Kühlbrandt, 2015). The high selectivity to ions of MIM creates a membrane potential across the MIM with an amplitude of about 180mV (Kamo et al., 1979).

The MOM and MIM delimit three compartments inside each mitochondrion. The innermost part surrounded by the MIM is the mitochondrial matrix. The space between the MOM and MIM is the intermembrane space (IMS). Unlike the MOM, MIM forms special membrane invaginations, extending deeply into the matrix, called cristae. The cristae define a particular compartment called the crista lumen. The crista lumen is isolated from the rest of the IMS by cristae junctions that are narrow constrictions. Most of all components of the electron transport

chain and the Adenosine Triphosphate (ATP) synthase are assembled at the membrane of the mitochondrial cristae (Kühlbrandt, 2015). The formation and remodeling of mitochondrial cristae are modulated by the optic atrophy-1 (Opa1) and the complex mitochondrial contact site and cristae organizing system (MICOS). Opa1, a dynamin-like GTPase, oligomerizes between its transmembrane and soluble form and brings two points of cristae junction into juxtaposition (Frezza et al., 2006). The MICOS complex regulates the formation of the cristae junctions and mediates the contact between the MOM and the MIM.

1.2.5.2. MOMP: release of harmful molecules

MOMP is a process that increases the MOM permeability to large molecules. It involves the formation of protein-based pores through the oligomerization of activated Bax and Bak. This process is relatively rapid (just a matter of seconds) and requires approximately 5 minutes to be completed (Rehm et al., 2009).

When the MOM is permeabilized, it was thought that the IMS proteins are freely released to the cytosol. Nonetheless, recent findings suggest that, following the pore formation, the mitochondrial membrane needs to be remodeled to promote efficient IMS protein release (Frezza et al., 2006; Prudent et al., 2015b).

In addition, MIM is also permeabilized following the MOMP, allowing the release of mitochondrial DNA (mtDNA) into the cytosol. This process is mediated through Bax/Bak macropore (McArthur et al., 2018; Riley et al., 2018). A recent study from Garcia-Saez team suggests that the protein Bak, rather than Bax, accelerates mtDNA release (Cosentino et al., 2022).

In the following, I will highlight some IMS proteins documentedly released during MOMP.

Cytochrome c

Cytochrome c is a highly conserved hem protein with a small size of about 12kDa. It plays a crucial function as a mobile electron carrier in the electron transport between complexes III and IV of the mitochondrial respiratory chain (Pérez-Mejías et al., 2019). As a component of the electron respiratory chain, cytochrome c is thought to be more distributed in the isolated cristae lumen (Kühlbrandt, 2015). Cytochrome c is rather mobile in the IMS, with only a small fraction bounded to the cardiolipin. That may explain why pore formation alone is not sufficient to trigger efficient cytochrome c release.

Following MOMP, mitochondria cristae are remodeled: individual cristae are fused, and cristae junctions are widened (Scorrano et al., 2002; Germain et al., 2005; Frezza et al., 2006). tBid has been found to weaken oligomers of Opa1, therefore, widen the cristae junctions before cytochrome c release (Frezza et al., 2006). Recent findings hint the cooperation between mitochondria and ER to induce the cristae remodeling upon apoptotic stimuli. A platform connecting ER and mitochondria *via* Drp1 oligomerization is essential to calcium and lipid transfer from the former to the later organelle, which is critical for cristae remodeling (Prudent et al., 2015b).

In the cytosol, cytochrome c binds to Apoptotic protease-activating factor 1 (Apaf-1), which enhances Apaf-1 oligomerization to form apoptosome (Zou et al., 1999). This complex is essential to activate caspase-9 and trigger apoptosis.

Second mitochondria-derived activator of caspase (SMAC)

SMAC, also called DIABLO, is an IMS protein that regulates the lipid synthesis of mitochondria (Paul et al., 2018). During MOMP, SMAC is liberated into cytosol simultaneously with cytochrome c. In the cytosol, it enhances apoptosis reaction by neutralizing the inhibitors of apoptosis protein (IAPs), including the X-linked inhibitor of apoptosis protein (XIAP), cellular IAP1 (cIAP1), and cIAP2 (Du et al., 2000; Verhagen et al., 2000).

Structural study indicates that only dimer forms of SMAC interact with XIAP as mutations that disrupt the dimerization capacity decrease its pro-apoptotic activity. Interaction with XIAP is mediated by the N-terminal motif sequence AVPI (Ala-Val-Pro-Ile). This motif binds to BIR2 and BIR3 of XIAP and releases caspase-3 and caspase-9, respectively (Chai et al., 2000; Srinivasula et al., 2001).

Omi/HtrA2

Omi (also known as HtrA2) is a serine protease principally localized in the IMS. Like SMAC/Diablo, OMI/HtrA2 possesses an IAP-binding motif (AVPI) at the N-terminus. During MOMP, it is released into cytosol with SMAC/Diablo and liberates caspases from their inhibitors (Hegde et al., 2002). Moreover, Omi/HtrA2 may proteolytically cleave IAPs through its protease activity (Yang et al., 2003).

Others

Among IMS proteins found in the cytosol due to MOMP, there are also endonuclease G and apoptosis-inducing factor 1 (AIF-1) (Susin et al., 1999; Li et al., 2001). Subsequently, they

translocate into the nucleus and induce DNA fragmentation and chromatin condensation independently of caspase.

1.2.5.3. MOMP – a no-return point of apoptosis?

MOMP was considered a no-return point of cell death. MOMP leads to cytochrome c release, which launches the caspase activation cascade. Whereas caspase-3 mediated damaging process is considered the major pathway, cells engage to death also *via* caspase non-dependent pathways in the context of MOMP. This phenomenon is conceivably due to the loss of mitochondrial functions or/and release of “cell toxic” agents from mitochondria upon MOMP (Tait and Green, 2008).

Disruption of MOM induces the leak of respiration chain components and regulatory factors, including cytochrome c and AIF. Concurrently, the mitochondrial potential $\Delta\Psi_m$ gradually drops, which represses ATP production (Ricci et al., 2004). Besides, other agents that are destructive to other cell components are also found in cytosols upon MOMP. Endonuclease G (EndoG) is a predominant endonuclease in the mitochondrial inner membrane. Following MOMP, EndoG is leaked into the cytosol and then translocated into the nucleus, where it potentially degrades nuclear DNA (Li et al., 2001).

Nonetheless, some evidence shows that, in certain situations, a cell is capable of escaping cell death destiny induced by MOMP. This phenomenon was first described in neurons. Indeed, in sympathetic mature neurons, nerve growth factor (NGF) withdrawal induces MOMP followed by cytochrome c release. However, mitochondria can recover from cytochrome c loss when NGF is replenished before $\Delta\Psi_m$ is totally dropped (Martinou et al., 1999; Deshmukh et al., 2000). Partial MOMP can also be produced *in vitro* by the treatment with a sub-lethal dose of BH3 mimetics (Ichim et al., 2015). These observations challenge the traditional notion of MOMP as the ultimate point of apoptosis.

The term “incomplete MOMP” was first utilized for a phenomenon in which MOMP does not lead to cell death. This phenomenon has been described in both physiological and pathological contexts (Ichim et al., 2015; Brokatzky et al., 2019; Berthenet et al., 2020), in both post-mitotic and proliferating cells (Martinou et al., 1999; Colell et al., 2007; Yosefzon et al., 2018).

There are two main scenarios of incomplete MOMP (iMOMP) (Kalkavan and Green, 2018). In certain cases, whereas MOMP occurs widespread in the absence of cell death, some intact mitochondria are escaped and play a role as “remain seeds” to re-establish replacing population. In another case, MOMP happens only in a minor mitochondria population, leading to non-lethal

caspase activation (Figure 7). In both cases, it induces the activation of caspase-3 that may produce critical cellular modifications.

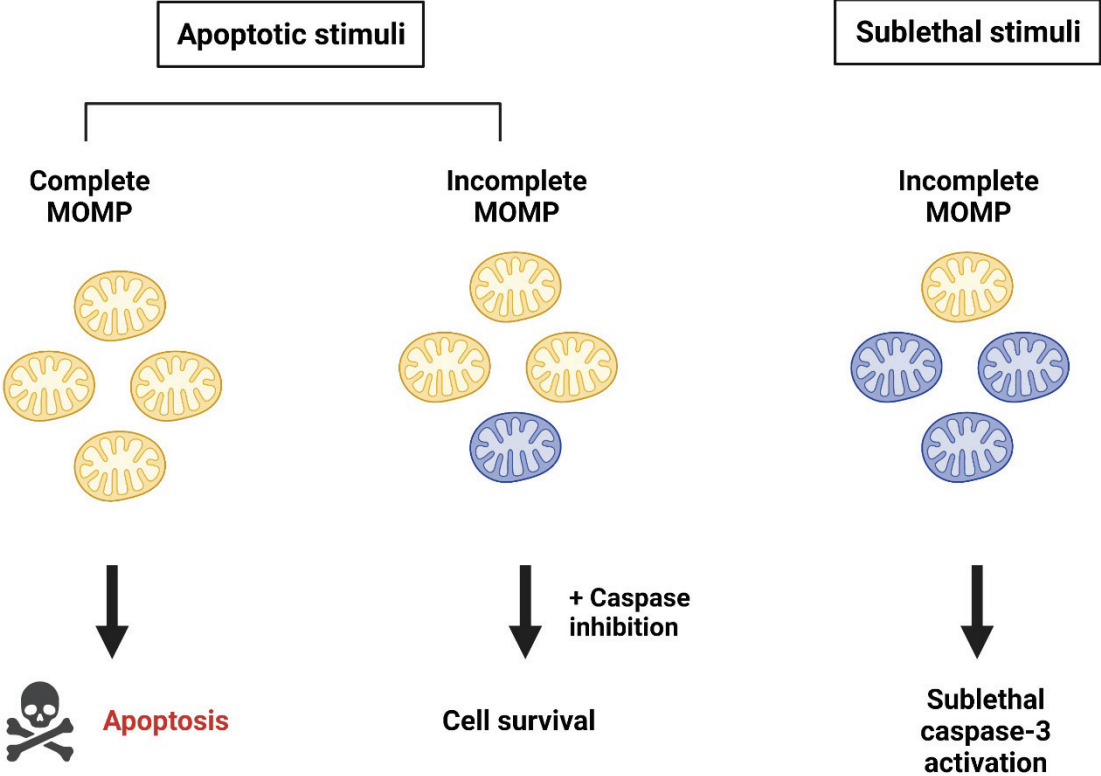


Figure 7. Overview of MOMP scenarios

The permeabilized mitochondria are represented in yellow, and the healthy ones in blue. In response to the apoptotic stimuli, MOMP can occur completely in the mitochondrial population and induce apoptosis. In some exceptional conditions, a minority of mitochondria are preserved contact. Cells can survive in these contexts if caspase activity is inhibited. In the third scenario, a minority of mitochondria are permeabilized in response to sublethal stimuli. This condition does not lead to cell death but activates sublethal caspase activation. Adapted from (Kalkavan and Green, 2018).

1.2.5.4. Incomplete MOMP: physiological contexts

iMOMP is rather a novel concept and has been described recently with the emergence of novel technologies. However, the involvement of sub-lethal caspase-3 activation in physiological processes has been studied for two decades. Indeed, non-apoptosis-related caspase-3 activation has been detected during the differentiation of multiple cell types. It was first observed in the differentiation of lens epithelial cells and erythrocytes, during which caspase activity is required for the enucleation of their cytoplasm (Ishizaki et al., 1998; Zermati et al., 2001; Carlile et al., 2004). This was considered a “benign” form of apoptosis, which was used to “destroy” some intracellular organelles or structures with “delayed” cell death. In later publications, caspase-3 activation was also documented in the long-lived cells. According to Fernando and colleagues, caspase-3 promotes myogenesis *via* proteolytic activating the protein mammalian sterile twenty-like kinase (Mst1), which, in turn, activates the p38 MAPK pathway (Fernando et al., 2002).

Recent evidence suggests that iMOMP plays an important role in regulating immune responses to microbial infection (Brokatzky et al., 2019). On the one hand, MOMP leads to mtDNA release into the cytosol, activating the cyclic guanosine monophosphate/adenosine monophosphate synthase/stimulator of interferon genes (cGAS/STING) pathway and promoting the interferon production (McArthur et al., 2018; Riley et al., 2018). On the other hand, caspase-activated DNase also induces interferon secretion via the STING pathway (Brokatzky et al., 2019). Nonetheless, in contrast, full activation of caspase-3 suppresses the cGAS/STING activation, suggesting that iMOMP is essential to regulate innate immune responses (Rongvaux et al., 2014; White et al., 2014).

Non-lethal caspase-3 activation is also detected in the neuron, a very long-lived cell type. It was first described in an axon fine-tuning process called axon pruning. This process occurs at the late embryonic or early postnatal stage to remove collateral extensions with improper connectivity. Caspase-3 modulates the axon pruning of neural cells of the peripheral nervous system (PNS) (Nikolaev et al., 2009; Simon et al., 2012; Cusack et al., 2013; Unsain et al., 2013). Otherwise, the role of caspase in axonal network fine-tuning in the central nervous system is less established. Recently, Gu et al. reported local caspase-3 activation in the axon of spinal cord neurons of post-natal mice without detectable signals in the cell body. Inhibition of caspase-3 activation by *bax/bak* double KO (DKO) led to a less tailored network of neuron branches, resulting in corticospinal circuit anomalies and impaired skilled movement in adult mice (Gu et al., 2017). Caspase-3 has been found to refine more “delicate” structures of neurons

as protein structure at the synapse terminal during synapse maturation. In the PNS, caspase-3 fine-tunes the post-synaptic neurotransmitter receptor network. During development, the nicotinic acetylcholine receptor (AChR), produced abundantly at the early stage, is partly eliminated *via* caspase-3 mediated Dishevelled (Dvl1) activation (Wang et al., 2014).

Even confined and dynamic signal of active caspase-3 has not yet been detected in the synapse by live imaging, an emergence of studies demonstrates the involvement of caspase-3 in synaptic functions and plasticity. The role of caspase-3 is highlighted in a process named long-term depression (LTD). It involves synaptic strength reduction in response to repeat and durable neural stimulations and is widely associated with spatial learning, memory formation, and learning processes (Whitlock et al., 2006). This phenomenon is well described in the glutaminergic synapse. Upon long-term exposure to glutamate, caspase-3 modulates post-synaptic structure, including α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPA) decrease at synapse surface and dendritic spine remodeling. Caspase-3 promotes AMPAR internalization through a not yet defined mechanism and also directly cleaves AMPAR subunits (Chan et al., 1999; Li et al., 2010; Gibon et al., 2016; Henson et al., 2017). Besides, caspase-3 induced dendritic spine density reduction and spine shape modification into a more thin long-form (Guo et al., 2016).

All studies mentioned above demonstrated the non-apoptotic roles of caspase-3 using pharmacological and/or genetic approaches. Active caspase-3 signals were rarely directly detected, even shown in rare cases, only in fixed cell cultures or tissues. That induced a lack of comprehension of the dynamics of non-canonical caspase-3 activation and omitted the slight signals, which might be under the detectable threshold of these methods. An adaptive and more sensitive tool to follow the real-time kinetics of caspase-3 activation in live cells in general and in developing neural cells, in particular, has become an urge in order to deepen our knowledge about the roles as well molecular mechanisms of this phenomenon.

With advances in fluorescence-based biosensors, our knowledge gap started to fill. Two prominent indicators of caspase activity have been developed, the caspase-3 activity indicator (C3AI) and the fluorescence resonance energy transfer (FRET)-based sensor for activated caspase-3 (SCAT3) monitoring. C3AI is a switch-on fluorescent-base indicator containing a circularly permuted non-fluorescent Venus. When the linker bearing a sequence of Asp-Glu-Val-Asp (DEVD) is cleaved due to caspase-3 activity, the indicator rapidly becomes fluorescent, reflecting caspase activation (Zhang et al., 2013). SCAT3 contains a peptide sequence of the enhanced cyan fluorescent protein (ECFP) used as the donor coupled with the

Venus used as the receiver. Two fluorescent proteins are linked by the DEVD peptide sequence (Takemoto et al., 2003). When caspase-3 is active, two fluorescent proteins are separated, disrupting the energy transfer and reducing the emission ratio between the receiver and the donor. Thus, caspase-3 activity is reported *via* Venus/ECFP emission ratio. As a FRET-based method, SCAT3 is more sensitive to small changes in caspase-3 activity without potential bias from background effects. This construction was used in the study of Campbell and Okamoto, in which they reported a transient slight caspase-3 activation at the branching point of the immature retinal ganglion cells (RGC), recorded shortly before or immediately after the formation of a newly tip (Campbell and Okamoto, 2013). This result supports the physiological role of caspase-3 in the axon branching and pathfinding process in the developing neurons, which has been hinted in the other studies (Verma, 2005; Pinan-Lucarre et al., 2012; Katow et al., 2017).

1.2.5.5. Incomplete MOMP in the pathological conditions

Cancer

To promote their growth and transformation, cancer cells need to block apoptosis. Resensitizing tumor cells to apoptotic stimuli is a prominent therapeutic strategy. However, cells may still escape their perishing fate in certain conditions. In fact, under sub-lethal stress, MOMP may occur incompletely, allowing some mitochondria to remain unpermeabilized (Tait et al., 2010; Ichim et al., 2015). However, the survival cells bear unrepairable cellular damage, especially nuclear and mitochondrial DNA impairments. This genomic instability caused by non-lethal caspase-3 is responsible for cancer relapse, metastasis, and therapeutic resistance *via* boosting carcinogenesis and tumor invasiveness (Liu et al., 2015; Berthenet et al., 2020).

Neurodegenerative disorders

Neurodegenerative disorders, including Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), are caused by the loss of a particular neuron population *via* apoptosis. These diseases are often the result of mutations, acquired or hereditary, in one or several genes leading to structural or functional modifications and triggering cell death (Mattson, 2000; Gorman, 2008). The lesions provoked by the neuron loss engender the selection of disease symptoms on the motive and cognitive functions. Caspase-3 activation is common in these neurodegenerative diseases as a marker of apoptosis (Mattson, 2000).

Interestingly, multiple proteins linked directly to the pathology of these diseases are also substrates of caspase-3. Recent findings on this matter are summarized below.

Alzheimer's disease

Alzheimer's disease (AD) is a common disorder of cognitive function loss. Its prevalence increases with aging. AD pathology is characterized by the overproduction of amyloid-beta ($A\beta$) plaques or/and aggregation of tau to form neurofibrillary tangles (Bloom, 2014).

$A\beta$ is a mix of 38 to 43 amino acid peptides derived from its large precursor termed β -amyloid precursor protein (APP). APP is a transmembrane protein type I, ubiquitously expressed in the neurons. In non-pathological conditions, APP undergoes a series of cleavage by α - and β -secretase that produces non-toxic soluble fragments into the extracellular matrix (O'Brien and Wong, 2011). In contrast, if the sequential cleavage of APP is initiated by β -secretase instead of α -secretase, proteolytic cleavage by γ -secretase will occur. This abnormal process produces pathogenic $A\beta$ peptide that then aggregates to form insoluble $A\beta$ plaques. The excess of $A\beta$ production in the early-onset genetic-related cases is usually linked to mutations in the APP gene itself or two other genes coding for presenilin-1 and -2, components of γ -secretase (Zhang et al., 2011).

Molecular mechanisms linking presenilin-1 and -2 to the overproduction of $A\beta$ have not been well understood. Notwithstanding, mutations in presenilin-1 and -2 present the most common cause of autosomal dominant AD. Data from in vitro and in vivo from *presenilin* KO mice show that loss of presenilin induces anomalies of γ -secretase functions (Elder et al., 2010). Presenilin is likely required for proper γ -secretase cleavage of APP. Interestingly, both presenilin-1 and -2 can be cleaved by caspase-3 (Kim et al., 1997).

Another potential target of caspase-3 during the AD is Golgi-associated, gamma adaptin ear containing ARF binding protein 3 (GGA3). GGA3 is an adaptor protein interacting with β -secretase, critical for the sorting, trafficking, and degradation of β -secretase. It is inactivated by caspase-3-induced cleavage (Tesco et al., 2007). That means under caspase-3 activation, β -secretase is stabilized, enhancing the production of $A\beta$.

Noteworthy, active caspase-3 is likely to accumulate in the synapse fraction of AD patients' brains (Louneva et al., 2008). Altogether, these observations raise a question if, at the early stage of AD, caspase-3 is locally activated, notably in the synapse, which enhances the progress of the disease.

Parkinson's disease

Parkinson's disease (PD) is a motion disturbing disease created by dopaminergic neuron loss in the substantia nigra region of the brainstem (Dauer and Przedborski, 2003). Parkin, a ubiquitin E3 protein ligase, is important to the survival of the dopaminergic neuron population in the brainstem. Loss-of-function mutation of Parkin is a common cause of PD (Kilarski et al., 2012). Parkin also possesses a cleavage site for caspase-3 and is proteolytically cut during apoptosis (Kahns et al., 2002).

Huntington's disease

Huntington's disease (HD) is a complex neurodegenerative pathology characterized by cognitive degeneration, movement disorder, and personality changes. This disease is caused by inherited mutations in the gene coding for protein Huntingtin (HTT), which supplies the N-terminus of protein with repeated CAG sequence. It results in an abnormal polyglutamine sequence at the N-terminus of HTT (Saudou and Humbert, 2016). The mechanism of HD progression remains unclear as HTT is a ubiquitous protein with a complex range of functions (Cattaneo et al., 2005).

Interestingly, HTT contains multiple consensus caspase-3 cleavage sites. Both wild-type and HD mutant forms of HTT are cleaved by caspase-3 (Kim et al., 2001; Wellington et al., 2002). However, cleavage in mutant HTT results in toxic fragments. N-fragment containing polyQ sequence translocates into the nucleus, leading to transcriptional deregulation and neural death (Saudou et al., 1998). It also induces signaling dysregulation, mitochondria dysfunction, and autophagy (Borrell-Pagès et al., 2006). Recent findings show that even C-terminal fragment from proteolytic cleavage is also harmful to the host cell. This fragment inactivates Dynamin 1 at the ER, which results in ER perturbation and cell death (El-Daher et al., 2015). Remarkably, caspase-related fragments are present in the brain of HD patients in the early stage, before neurodegeneration onset (Wellington et al., 2002). Inhibition of caspase-3 attenuates neural toxicity and cell death (Wellington et al., 2000).

Altogether, these findings hint potentially a novel role of caspase-3 in the progression of neurodegenerative diseases. Activation of caspase-3 may not only result from pathology development, the marker of neural loss but also functions as a pathogenic factor. Slight, locally active caspase-3 signals caused by cellular stress at early-stage lesions may create a positive feedback loop that amplifies and aggravates cellular damages.

1.2.5.6. Mechanism of iMOMP

Permeabilization of a population of mitochondria

The molecular mechanisms of iMOMP remain elusive. Tait and his colleagues propose a noticeable hypothesis based on the heterogeneous distribution of Bcl-2 anti- and pro-apoptotic proteins at the mitochondrial. According to this model, Bcl-2 proteins are not disposed equivalently between individual mitochondria. Consequently, mitochondria harboring high amounts of anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, would be protected from Bax and Bak-induced permeabilization, whereas mitochondria deprived of apoptosis inhibitors would be privileged prey for Bax and Bak (Tait et al., 2010). Recently, the inter-mitochondrial heterogeneity of anti-apoptotic Bcl-2 proteins has been directly visualized using CRISPR-Cas9 generated knock-in strategy. In these experiments, the sequence coding for the fluorescent protein mCherry was put in the N-terminus part of *Bcl2*, *Bclx*, and *Mcl1* loci (Cao et al., 2022). In such a way, Cao and colleagues demonstrated that these three anti-apoptotic proteins have heterogeneous distribution at the mitochondrial level. Interestingly, this heterogeneity was abolished in hyper-fused mitochondria suggesting that an “internal” Bcl-2 circuit at MOM may exist to obtain a more “homogenous” anti-apoptotic distribution and efficient protection.

The mechanism allowing such heterogeneity is still unclear. It might involve a signal to recruit Bax/Bak to a specific population of mitochondria and/or a signal to shift anti-apoptotic proteins either to another mitochondrial pool or the cytosol. In line with this, the retromer is a system conceivably able to mediate the translocation of anti-apoptotic proteins to distinct mitochondrial populations. Indeed, a recent study showed that Bcl-xL forms a complex with VPS35/VPS26 retromer proteins. This complex promotes the mitochondrial localization of Bcl-xL (Farmer et al., 2019). Thus, a signal may be present at the mitochondria to recruit this Bcl-xL-retromer complex. Anti-apoptotic proteins might also be eliminated from mitochondria through Mitochondrial-anchored protein ligase (MAPL)-mediated mitochondrial-derived vesicles, a structure transferring MOM proteins from the mitochondria to the peroxisome (Neuspiel et al., 2008).

In mature neurons, moderate Bax activation is observed in response to N-methyl-D-aspartate (NMDA) receptor-dependent long-term depression. Interestingly, in this case, translocation of Bax to mitochondria could not be detected, suggesting that only a minor fraction of Bax, already present at the level of the mitochondria, was activated. Alternatively, Bax translocation might be too low to be detected with current methods (Jiao and Li, 2011).

Recent work also suggested a potential role of mitochondria network dynamics on iMOMP regulation. In this study, iMOMP was observed selectively in small fragmented mitochondria resulting from Drp1-mediated fission. Accordingly, mitochondrial fusion induced by Mitofusin inhibits the phenomenon of iMOMP (Cao et al., 2022). This observation may give an explanation for local caspase-3 activation in the neuron projections. To freely enter and travel along the narrow space of the axon and the dendrites, mitochondria are scissored into small individuals. In addition, the fusion rate in the axon is kept low to maintain this fragmentation-preferred condition. That clearly creates an environment to produce and maintain inter-mitochondria Bcl-2 heterogeneity required to trigger iMOMP.

Preservation of healthy mitochondria under iMOMP

For a cell to survive and recover under iMOMP, a population of mitochondria must be protected and preserved. These remaining healthy mitochondria play as the seeds to replenish and recreate the mitochondrial network (Tait et al., 2010).

The permeabilization of mitochondria has been described as a rather rapid and complete process (Goldstein et al., 2000). Actually, the propagation of cytochrome c-release waves has been reported during apoptosis (Lartigue et al., 2008; Cheng and Ferrell, 2018). Such propagation of cytochrome c into the cytosol seems to be independent of pro-apoptotic proteins Bax and Bak, and the exact mechanisms are still a matter of debate (Lartigue et al., 2008). While cytochrome c is unlikely to be capable of self-amplify its own release from neighboring mitochondria, two potential candidates were proposed: calcium signaling and the caspase pathway. Indeed, Ca^{2+} may play a role in this respect. Aside from cytochrome c and cognate apoptogenic agents, depolarized mitochondria initiate Ca^{2+} waves and trigger the depolarization of surrounding mitochondria *via* MPTP (Pacher and Hajnóczky, 2001; Ziegler et al., 2021). Cytochrome c also directly interacts with IP_3R and induces ER calcium efflux (Boehning et al., 2003). Concurrently, cytochrome c release activates effector caspases, including caspase-3 and caspase-7, that process and redistribute certain BH3-only to the MOMP and amplify the phenomenon (Lartigue et al., 2008; Shelton et al., 2009).

Even though cytochrome c triggers the self-amplification loops of MOMP in an indirect way, rapid degradation of cytochrome c is presumably an efficient strategy to prevent the complete MOMP. Indeed, in post-mitotic neurons, an E3 ligase, referred to as Cullin 9 (CUL9) or Parkin-like cytoplasmic protein (PARC), was reported to mediate cytosolic cytochrome c degradation,

suggesting that the propagation of cytosolic cytochrome c may be blocked by proteasome-mediated degradation (Gama et al., 2014).

1.2.6. Regulation of Bcl-2 proteins by post-translational modification

The activity of Bcl-2 proteins is controlled by posttranslational modifications (PTMs), including phosphorylation, ubiquitination, and deamidation. Accordingly, these modifications represent a way for rapid regulation, either promoting or inhibiting the activities of Bcl-2 proteins.

Besides, Bcl-2 protein activities are also regulated by their binding partners. For instance, the anti-apoptotic protein Bcl-2 is inverted into a pro-apoptotic protein via binding to the nuclear hormone receptor 77 (Nur77). Indeed, Nur77 binds to the N-terminal loop of Bcl-2 and exposes its BH3 motif on the surface. This conformational modified Bcl-2 then behaves as a BH3-only sensitizer to activate Bax/Bak proteins (Lin et al., 2004). Bcl-xL activity is also regulated by its binding partner, E2F1. When accumulated at the mitochondrial, E2F1 stabilizes the mitochondrial localization of Bcl-xL and dampens its anti-apoptotic function (Vuillier et al., 2018).

Here, we review some documented PTMs of key Bcl-2 proteins focusing on the anti-apoptotic protein Bcl-xL.

1.2.6.1. Phosphorylation

Phosphorylation of anti-apoptotic protein Bcl-2 proteins

Phosphorylation of anti-apoptotic protein can promote or inhibit its effects depending on the position of phosphorylation. It has been shown that phosphorylation in the flexible loop situated between the BH3 and BH4 motifs is important for the anti-apoptotic function. This region is present in Bcl-2, Bcl-xL, and Mcl-1. Phosphorylation at the flexible loop of Bcl-2, especially at Ser70, is necessary for its full potent anti-apoptotic activity (May et al., 1994; Ito et al., 1997). Ser70 undergoes a dynamic process of phosphorylation and dephosphorylation mediated by protein kinase C α (PKC α) and protein phosphatase 2A (PP2A) (Deng et al., 1998). In various conditions, for instance, stimulation by paclitaxel or microtubule inhibitors, Bcl-2 can be phosphorylated on multiple sites of its flexible loop, including Ser70, Thr69, and Ser87. This hyperphosphorylation form of Bcl-2 also promotes its anti-apoptotic activities (Deng et al., 2004).

Unlike Bcl-2, phosphorylation at the unstructured loop induced by taxol treatment inhibits the anti-apoptotic function of Bcl-xL (Basu and Haldar, 2003). This modification is mediated by

Jun N-terminal Kinase (JNK). Later, phosphorylation at this site is shown to play a role in the G2 checkpoint that induces G2 arrest in response to DNA damage (Wang et al., 2012a). In this context, this phosphorylation is induced by other kinases, including Mitogen-activated protein kinase (MAPK9)/JNK2 or even Polo-like kinase (PLK) 1/3. Other phosphorylation sites identified out of the flexible loop include Thr47 and Thr115 in response to DNA damage by JNK (Kharbanda et al., 2000), Ser14 (residing in the BH4 motif) by oxidative stress-activated Mst1 in cardiac myocytes (Del Re et al., 2014), and Ser73 by cyclin-dependent kinase 2 (Cdk2) in the kidney cell (Seng et al., 2016). All these phosphorylations perturb the anti-apoptotic activity of Bcl-xL and result in the activation of apoptosis.

Mcl-1 has a PEST domain at the N-terminus, which is a region rich in Proline, Glutamate, Serine, and Threonine, and contributes to the shorter half-life of Mcl-1 (2-4 hours). Accordingly, PEST region phosphorylation regulates the stability of Mcl-1 (Domina et al., 2004). Mcl-1 can be phosphorylated at Thr92 and Thr163 by Ras-Raf-extracellular signal-regulated kinase (ERK) to promote its stability and anti-apoptotic potential (Ding et al., 2008) or at Ser159 by glycogen synthase kinase 3 (GSK-3) leading to its degradation (Maurer et al., 2006; Wang et al., 2013). Recent findings presented a novel degradation-independent function of Mcl-1 phosphorylation induced by histone deacetylase inhibitors (HDACi). Mcl-1 phosphorylation induced by HDACi through GSK inhibits its interaction with BH3-only protein, sensitizing it to apoptosis (Tong et al., 2018).

Phosphorylation of anti-survival Bcl-2 proteins

Bax phosphorylation usually prevents its activation and its apoptotic function. Phosphorylation at Ser184 in its C-terminal TM motif by protein kinase B (PKB) dampens its heterodimerization with Mcl-1, Bcl-xL and A1, therefore decreasing its translocation to the mitochondria and thus inactivating (Gardai et al., 2004; Garenne et al., 2016). This phosphorylation is also mediated by the protein kinase C ζ (PKC ζ) (Xin et al., 2007). Reversely, dephosphorylation of this site by PP2A disrupts the interaction between Bax and Bcl-2 and induces the activation of Bax at MOM (Xin and Deng, 2006). Furthermore, such phosphorylation is likely to convert Bax into anti-apoptotic protein as it promotes Bax binding to activator BH3-only proteins in solution and prevents their pro-apoptotic function as described in Bcl-2, Bcl-xL, and Mcl-1 (Kale et al., 2018a). Conversely, Bax can be activated by phosphorylation at Ser163 by GSK3 β or JNK (Linseman et al., 2004; Kim et al., 2006a).

In healthy cells, Bak is also kept under inactive form by phosphorylation. Bak activation usually involves a dephosphorylation process. In the initial step of activation, Bak undergoes dephosphorylation at Tyr108, which is necessary for exposure of its N-terminal and consecutive oligomerization. Otherwise, this modification is not sufficient for the entire activation of Bak (Fox et al., 2010). The second dephosphorylation site required for its oligomerization is Ser117, which is located in the hydrophobic groove. This dephosphorylation, mediated by PP2A, gives activator BH3-only proteins access to the hydrophobic groove of Bak, which induces conformational changes and oligomerization (Azad et al., 2012). A year later, it was shown that the dephosphorylation of Bak on Tyr110 was also important its activation (Azad and Storey, 2013).

Recent studies show that Bok, as well as Bax and Bak, are targets of the kinase GSK3 α/β . The phosphorylation induced by GSK3 confines the level of protein Bok (Onyeagucha et al., 2017). However, the phosphorylation sites and their mechanism for protein regulation remain to be discovered.

1.2.6.2. Ubiquitination and proteasomal degradation

Ubiquitination is the process in which ubiquitin is added to the substrate protein. It allows directing the targeted protein to the degradation process mediated by the ubiquitin-proteasome system (Ciechanover and Schwartz, 1998). Besides its role in protein quantity regulation, it also plays major roles in non-proteolytic processes, including assembly of a multiprotein complex, protein internalization, and DNA repair (Popovic et al., 2014). The Bcl-2 protein level is also under regulation by ubiquitination-proteasomal degradation.

Ubiquitination-mediated degradation of pro-survival Bcl-2 proteins

The first study put in evidence the regulation of Bcl-2 *via* ubiquitination mediated proteasome degradation was realized by Dimmeler and his colleagues. This study suggests that Bcl-2 degradation is induced by ubiquitination of putative MAP kinase phosphorylation sites of Bcl-2, including Thr56, 74, and Ser 87. Indeed, when these sites are phosphorylated, the degradation of this anti-apoptotic is inhibited (Dimmeler et al., 1999). A recent study identified the implication of XIAP, a caspase ubiquitin-protein ligase, in the degradation of Bcl-2. In fact, this process involves a mitochondrial protein, ARTS. Upon apoptotic stimuli, ARTS recruits XIAP to mitochondria and forms a ternary complex with Bcl-2 enhancing the ubiquitination of Lys17 and thus its degradation (Edison et al., 2017). A recent study showed that Parkin, an E3 ubiquitin ligase, mediates mono-ubiquitination of Bcl-2, stabilizing the protein and increasing

its amount. This effect, which is opposite from the one of poly-ubiquitination, is thought to be the mechanism of autophagy regulation by parkin (Chen et al., 2010).

The Bcl-xL expression level is strictly regulated by ubiquitin-mediated proteasome degradation. In response to apoptotic stimuli, the Bcl-xL level is down-regulated by the proteasome to trigger apoptosis (Park and Lee, 2009; Kajihara et al., 2014). It has been found that phosphorylation of Ser62 triggers its polyubiquitination and, therefore, degradation. Protein phosphatase 6 opposes this action of JNK by dephosphorylating this Ser (Kajihara et al., 2014). Parkin also binds directly to Bcl-xL. However, this interaction enhances Bcl-xL ubiquitination-mediated degradation (Gong et al., 2016). Furthermore, Bcl-xL is also regulated by ERAD. RNF183, a novel membrane-spanning RING finger protein, is induced by ER stress. This upregulation increases apoptosis through polyubiquitination-regulated degradation of Bcl-xL (Wu et al., 2018).

Hitherto, four different E3 ubiquitin-ligase have been found to ubiquitinate and regulate the level of protein Mcl-1 directly. The first identified is Mcl-1 ubiquitin ligase 3, or Mule. Five lysine sites have been described as Mule targets, including Lys 5, 40, 136, 194, and 197. This process is indispensable for DNA damage-induced apoptosis (Zhong et al., 2005). Other E3 ligases implicating in Mcl-1 proteasome-mediated degradation are SCF^{β-TrCP} (β-TrCP for beta transducing repeats containing protein) (Ding et al., 2008), SCF^{Fbw7} (Fbw7 for F-box and WD repeat domain-containing protein-7) (Inuzuka et al., 2011), and Trim17 (Tripartite motif-containing 17) (Magiera et al., 2013). The ubiquitination function of both β-TrCP and Fbw7 is dependent on phosphorylation induced by GSK3. Indeed, phosphorylation at Ser153 and/or Ser159 combined with Thr163 is necessary for the activity of the two ligases (Ding et al., 2008; Inuzuka et al., 2011). On the other hand, ubiquitination of Mcl-1 can be reversed by the deubiquitinase (DUB) called ubiquitin-specific peptidase 9 X-linked (USP9X) (Schwickart et al., 2010).

Ubiquitination-mediated degradation of pro-apoptotic multi-motif Bcl-2 proteins

Like Bcl-2 anti-apoptotic proteins, pro-apoptotic proteins are also under regulation of ubiquitin-mediated degradation. The proteasome inhibition leads to the accumulation of Bax in the mitochondria and the cytosol and subsequently induces cytochrome c release (Li and Dou, 2000). The ubiquitin ligase Parkin is also shown to ubiquitinate Bax and maintain its basal level (Johnson et al., 2012). A recent study suggests that, in response to mitophagy, Parkin also

inhibits Bax-mediated apoptosis in a ubiquitin-independent manner. The mechanism of this inhibition is still unknown (Bernardini et al., 2019).

Parkin not only interacts with Bax but also with Bak. Upon mitophagy, Parkin ubiquitinates Bak at Lys113 residue in its hydrophobic groove surface. This ubiquitination impairs its activation process and inhibits the formation of oligomers (Bernardini et al., 2019).

Under normal conditions, Bok stability is dependent on its interaction with IP₃Rs. Unbound Bok is ubiquitinated and degraded by the proteasome (Schulman et al., 2016). Furthermore, Bok degradation is mediated by the ERAD system. It involves E3 ligase AMFR/gp78 and VCP/p97 (Llambi et al., 2016).

1.2.6.3. Deamidation

Deamidation is a process in which the side-chain amino group (-NH₂) of asparaginyl and glutaminyl residues is removed to form aspartyl and glutamyl, respectively. This process alters the structure and function of proteins. Regulation by deamidation is likely an exclusive feature of Bcl-xL as it contains four Asn residues in the flexible loop, unlike Bcl-2, which does not have any. Among these four Asn residues, two potential targets of deamidation are Asn52 and Asn66. Upon exposure to DNA damaging agents such as etoposide or cisplatin, Bcl-xL is deaminated at these two positions, which decrease its anti-apoptotic propriety (Deverman et al., 2002). Mutations converting asparagine into alanine residues increase Bcl-xL anti-apoptotic function.

The decrease of Bcl-xL anti-apoptotic activities induced by deamidation may be mediated in two ways. Firstly, deamidation at Asn52 and Asn66 leads to exposure of a PEST sequence, which is a target for calpain-mediated degradation. This process, therefore, diminishes the cellular amount of Bcl-xL (Dho et al., 2013). Secondly, it has been suggested that deamidation of the two mentioned residues generates iso-Asp52 and 66. This latter form of Bcl-xL losses its capacity to sequester its binding partners, including Bim and Puma (Zhao et al., 2007).

Deamidation of Bcl-xL may be a potential target for cancer therapy. Accordingly, deamidation is a mechanism for cancer cells to gain resistance to apoptosis (Takehara and Takahashi, 2003). This process can be regulated by retinoblastoma protein (Rb), a tumor suppressor. Rb suppresses the Bcl-xL deamidation and its calpain-mediated degradation. The activity of Rb is, in turn, regulated by its phosphorylation status. In fact, activation of p53 leads to the inactivation of cyclin-dependent kinase (Cdk), therefore keeping Rb under the hypophosphorylated form, the active form for its Bcl-xL deamidation inhibition (Deverman et al., 2002).

1.3. Caspase family – effectors of apoptosis

In metazoans, apoptotic mechanisms are evolutionarily conserved and allow multicellular organisms to preserve tissue homeostasis (Ameisen, 2002). Ced-3 was the first apoptosis executioner discovered in *Caenorhabditis elegans* (Ellis and Horvitz, 1986). Its first mammalian homologs, the interleukin-1 β -converting enzyme in humans and nedd-2 in mice, were described shortly after (Yuan et al., 1993). Homologs of these proteins were also found in insects, namely Dcp-1 and Drice in *Drosophila*. These proteins were then named caspases, which stands for Cysteine Aspartyl-Specific Proteinases, reflecting their specificity for sites containing aspartic acid peptide bonds, which they cleave through the cysteine present in their active site. So far, about 15 caspase family members have been described in humans and mice (Shalini et al., 2015). Of note, in mammals, certain caspases are also implicated in inflammation. In this respect, it has been found that a given caspase can contribute to either apoptosis or inflammation, but not both (Martinon and Tschopp, 2004; Shalini et al., 2015). In the limit of my thesis, I mention only the apoptotic caspases.

1.3.1. Classification and structure of apoptotic caspases.

Apoptotic caspases, including those in humans, are classified into two groups: the initiators (caspase-8, -9, and -10) and the executioners (caspase-3, -6, and -7). These proteases are synthesized as proteolytically inactive zymogens. The inactive linear caspases contain a pro-domain, followed by a large p20 subunit and a p10 small subunit (Figure 8). Two subunits are connected by a linker. Activation of the pro-caspase generally involves the removal of the two-subunit linker and the pro-domain. The two subunits are separated and then dimerized (Fuentes-Prior and Salvesen, 2004).

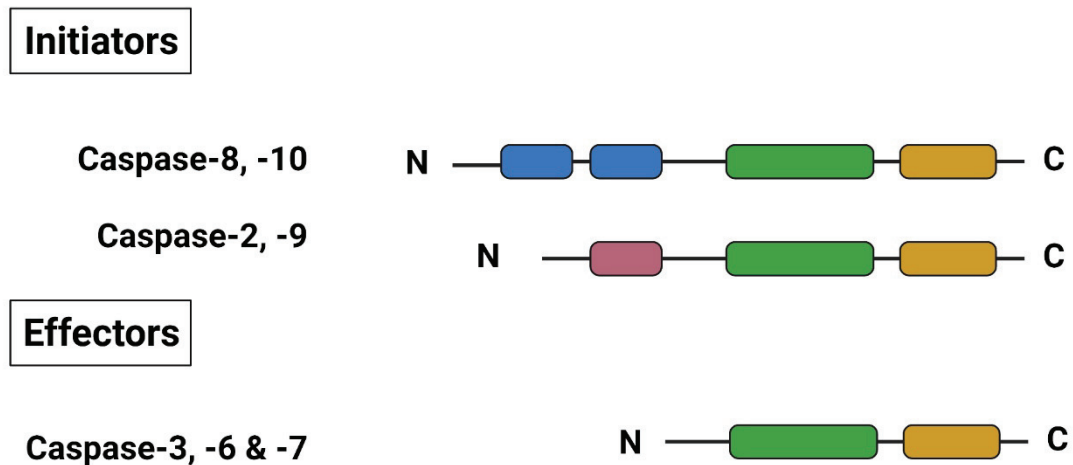


Figure 8. Domain structures of human apoptosis caspases.

Human apoptosis caspases are divided into two groups: initiators and effectors. All caspases possess a large subunit (green box) and a small subunit (yellow box). Initiator caspases have the DED-DED (blue box) or CARD (mauve box) domain at the N-terminus to interact with the caspase activation platform.

1.3.1.1. Initiator caspases

Initiator caspases include caspase-2, -8, -9, and -10. These members do not directly induce cellular modifications in apoptosis but rather function as sensors of apoptotic stress. Upon cell death stimuli, initiators, presenting as inactive zymogens, become active and activate the executioners.

The initiator caspases comprise a long pro-domain in the N-terminus, upstream of the conserved region encoding the large and small subunits of the active site. The pro-domain contains a specific sequence for adaptor protein binding, which is critical for the activation and catalytic activities of the host protein. Caspase-9 bears the caspase recruitment domain (CARD) motif, permitting it to interact with Apaf-1 (Chou et al., 1998). Caspase-8 and -10 possess the death effector domain (DED) motif, allowing their interaction with the Fas-associated *via* death domain (FADD) (Yao et al., 2007).

Initiator caspases can self-activate *via* autocatalysis. However, additional interactions with an activating protein platform are usually required to accelerate the activation process. For instance, the activation rate is several orders of magnitude higher when caspase-9 is docked in a supramolecular complex referred to as the apoptosome compared to the free form (Rodriguez

and Lazebnik, 1999). In this respect, two well-known activation platforms of initiator caspases are the death-inducing signaling complex (DISC) and the apoptosome complex.

Apoptosome comprises the apoptotic protease-activating factor 1 (Apaf-1), cytochrome c, and the cofactor dATP/ATP. Caspase-9 is recruited to the apoptosome through homophilic interaction between the CARD motif of caspase-9 with the one of Apaf-1 (Zhou et al., 1999). The pro-domain containing the CARD motif is unlikely to be cleaved during the activation process of caspase-9. After being processed, active caspase-9 is likely to continue binding to the apoptosome complex through the CARD motif and execute its catalytic activities in the holoenzyme complex with apoptosome (Rodriguez and Lazebnik, 1999; Li et al., 2017).

On the other hand, caspase-8 contains tandem DED motifs in its pro-domain and is activated by a different platform, the Death-inducing signaling complex (DISC). The DISC is composed of a transmembrane death receptor, such as Fas (also called CD95 or APO-1), the protein adaptor FADD, and caspase-8. When activated, Fas receptors cluster and recruit FADD *via* hemophilic interaction between the death domain (DD). Consecutively, pro-caspase-8 is recruited to the Fas-FADD complex *via* interaction between the DED-DED motif. As a result, the DISC brings multiple molecules of pro-caspase-8 together to enhance their activation *via* autocatalysis. That also favors the dimerization of caspase-8 (Oberst et al., 2010). Full activated dimer leaves the DISC complex and executes its activities in the cytosol.

The activation process of caspase-2 and -10 is less known. Even containing the CARD motif, caspase-2 activation is not mediated by apoptosome but by p53-induced protein with a death domain (PIDD) platform named PIDDosome (Tinel and Tschopp, 2004). The PIDDosome is composed of an adaptor protein RAIDD and the PIDD. RAIDD presents a CARD motif that recruits the caspase-2, but also a DD that is thought to bind to the PIDD. The structure and molecular mechanism of PIDDosome remain unknown. Caspase-10 is activated by the DISC complex as caspase-8, its function and downstream is otherwise unclear and controversial (Sprick et al., 2002; Horn et al., 2017; Mohr et al., 2018).

1.3.1.2. Executor caspases

Executor caspases are composed of caspase-3, -6 and -7. These proteins have a large subset of substrates and are responsible for almost cellular modifications during apoptosis.

Executor caspases have a short or even absent pro-domain. Unlike the initiators, executor caspases, lacking a proper pro-domain, can dimerize shortly after being synthesized. However, the linker region between the large and small subunits prevents their substrates access to the

catalytic center. Removal of the linker is performed by initiator caspases, such as caspase-8 and -9 induced conformational changes of the active site (Kumar, 2007).

1.3.2. Caspase substrates

Several hundreds of proteins are cleaved by caspases during apoptosis, but only a small proportion is considered biologically significant for cell remodeling (Julien and Wells, 2017). Among the most well-known targets of effector caspases are cytoskeletal proteins, including actin and tubulin (Mashima et al., 1999; Sokolowski et al., 2014), and nuclear envelope proteins, including lamins (Slee et al., 2001; Raz et al., 2006).

So far, an exhaustive view of the specific roles of each member of the caspase family is lacking. The precise map of their actual substrates also remains to be determined, although the preferred cleavage sites of each caspase were identified by proteomic studies. Caspase-3 and -7 are highly similar in terms of structure and substrate specificity. Both share the DEVD sequence as a preferential cleavage site. However, caspase-3 was more efficient than caspase-7 in cleaving well-known substrates such as Bid, XIAP, and caspase-6. Moreover, the amplification of the apoptotic cascade through the caspase-9 feeding loop depends on caspase-3 but not on caspase-7 (Walsh et al., 2008).

In the developing system, caspase-3 and -7 are the main caspase effectors. The redundancy of caspase-3 and -7 is clearly dependent on multiple factors, including the genetic background. *Casp3* null mice with 129/SvJ strain genetic background die at the embryonic state with hyperplasia in the brain (Kuida et al., 1996). However, under the C57BL/6 background, *casp3*-null mice are *viable* (Lakhani, 2006). *Casp7*-null mice also develop normally without any anomalies. The DKO mice of *casp3* and *casp7* under C57BL/6 background die rapidly after birth due to cardiac malformation.

Caspase-6 has VEHD as its preferential cleavage sequence, different from caspase-3 and -7 (Thornberry et al., 1997). The role of caspase-6 in apoptosis has not been well described. Active caspase-6 mediates nuclear lamin cleavage, inducing DNA fragmentation and nucleus shrinkage (Lazebnik et al., 1994; Takahashi et al., 1996). It has been shown to involve in stress-induced cell death in neurons (LeBlanc et al., 1999; Guo et al., 2006; Shabanzadeh et al., 2015). Noteworthy, recent findings connect caspase-6 to innate immune response and inflammasome activation (Zheng et al., 2020). That suggests a cross-talk may exist between two distinct groups of caspase.

In fact, the subset of substrates of effector caspase may depend on cellular context (Kuranaga and Miura, 2007). Indeed, during non-apoptotic caspase activation, cleavage of caspase targets remains strictly controlled in time and space, avoiding detrimental cell death. For instance, in erythropoiesis, the transcription factor GATA-1 is protected from caspase-mediated proteolysis by the chaperon protein Hsp70 (De Maria et al., 1999; Ribeil et al., 2007).

1.3.3. Caspase regulation

1.3.3.1. Caspase activation: the intrinsic and extrinsic pathways

Caspase activation is a general term used to indicate the cascade of activation leading to the ultimate activation of effector caspases; it generally mentions the activation of caspase3/7. As the executioners of apoptosis, whose activation can lead to broad and often non-reversible cellular damages, caspase activation is strictly regulated. On the other hand, caspase activation needs to be connected to a vast of cellular signaling to create appropriate responses to different stresses and conditions in which apoptosis is necessary.

The caspase activation cascade occurs *via* two broadly recognized pathways, referred to as extrinsic and intrinsic. In the extrinsic pathway, stimulation of death receptors such as Fas/Apo1 or TRAIL leads to the activation of initiator caspase-8 and downstream cleavage of effector caspase-3. The intrinsic pathway involves the mitochondria and notably the release of cytochrome c from the mitochondrial intermembrane space into the cytosol. Released cytochrome c binds to the Apaf-1 docking protein and facilitates the formation of the apoptosome complex that recruits and activates caspase-9. This caspase-9/Apaf-1/cytochrome c apoptosome complex is the holoenzyme form of caspase-9, which activates the caspase-3 apoptosis executioner by proteolytic cleavage (Hengartner, 2000).

The intrinsic pathway

The intrinsic pathway is triggered by intracellular stresses, such as DNA damage and metabolic stress. It is also named the mitochondrial pathway of apoptosis, which emphasizes the critical role of mitochondrial permeabilization in this pathway (Figure 9). Indeed, the most pivotal event is the release of cytochrome c due to the MOMP in response to apoptotic stresses (Westphal et al., 2014b). In the cytosol, cytochrome c binds to Apaf-1 to trigger its activation.

Apaf-1, identified as the central component of the apoptosome, is composed of three distinct functional domains: a CARD domain at the N-terminus, a regulatory region containing 12-13 times of WD40 repeat at the C-terminus, and a nucleotide-binding domain in the middle (Riedl and Salvesen, 2007). The regulatory domain keeps Apaf-1 at the inhibitory state. When

cytochrome c binds to the regulatory domain, it allows ATP or dATP to bind to the nucleotide-binding domain and induces conformational changes of Apaf-1 (Zhou et al., 2015). The activation is accomplished when seven individual Apaf-1/cytochrome complexes are oligomerized to form a ring-like complex, the apoptosome. The central ring of apoptosome contains seven CARD domains in juxtaposition. CARD domain recruits pro-caspase-9 to apoptosome and activates it (Cain et al., 2000). Consequently, the complex Apaf-1/caspase-9 acts as a holoenzyme to proteolytically activate caspase-3 and -7 (Rodriguez and Lazebnik, 1999).

Caspase-3 creates an amplification feedback loop to enhance the enzymatic cascade of activation *via* caspase-9 direct cleavage or caspase-inhibitor inhibitory cleavage (Fujita et al., 2001; Hörnle et al., 2011; McComb et al., 2019).

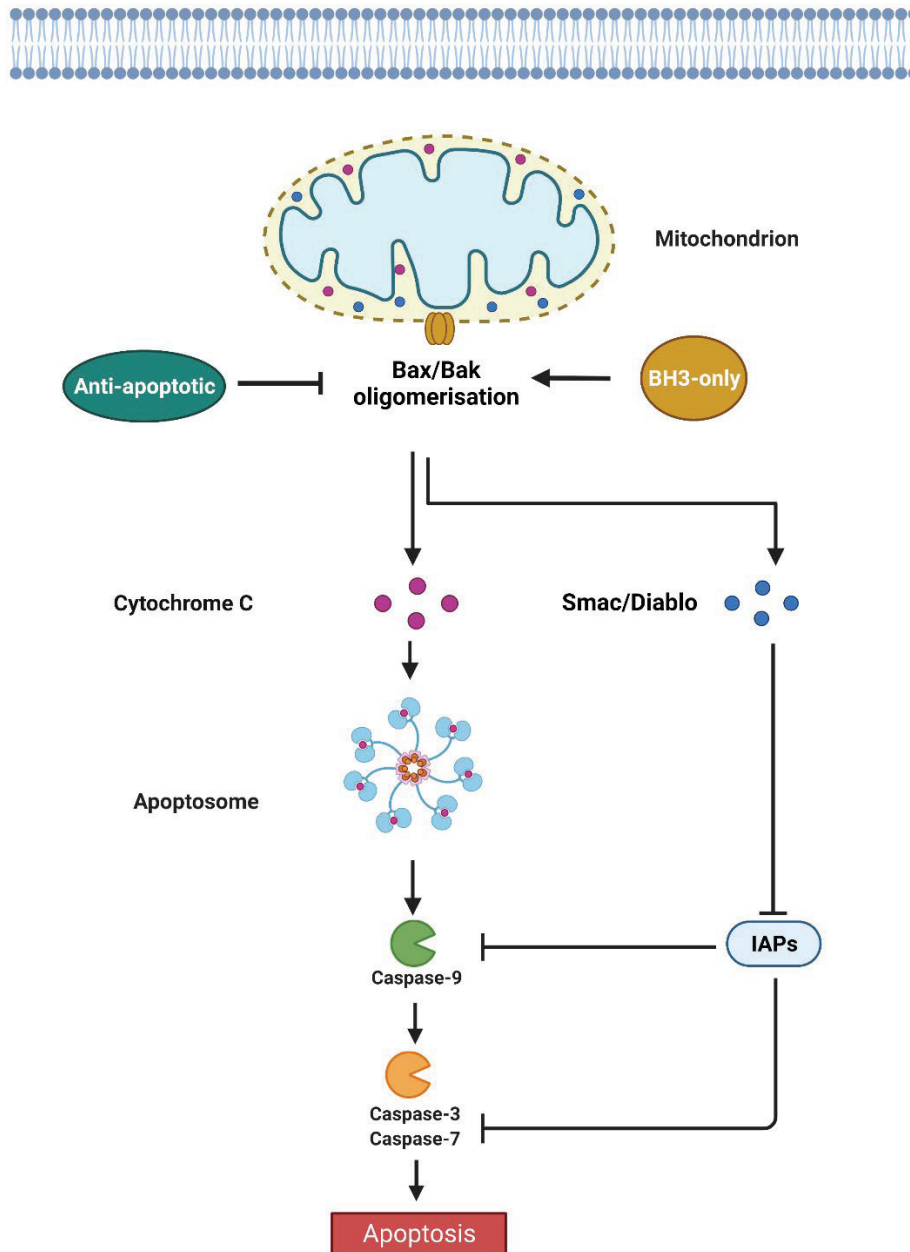


Figure 9. Schematic representation of the intrinsic pathway of apoptosis.

The intrinsic or mitochondrial pathway of apoptosis is triggered by intracellular stresses such as DNA damage. Upon stimuli, Bax and Bak proteins are activated and form the pore channel. This phenomenon is referred to as MOMP. MOMP induces the release of apoptogenic agents, including cytochrome c and Smac/Diablo. The former triggers the assembly of apoptosome that activates caspase-9 and then caspase 3/7. The latter inhibits the caspase inhibitors IAPs. Overall, caspase-3 and -7 execute the cellular modifications and lead to apoptosis.

The extrinsic pathway

The immune system uses the extrinsic pathway as a tool to eradicate infectious or transformed cells (Figure 10) (Wallach and Kang, 2018). It is mediated by death receptors, including Tumor necrosis factor receptor 1 (TNFR1), Fas receptor, and TNF-related apoptosis-inducing ligand receptor-1 and -2 (TRAILR1 and R2). The death receptors are activated by their corresponding ligands. The majority of cell death ligands are type-II transmembrane proteins. Death ligands can be cleaved and released as soluble cytokines in some cases (Guicciardi and Gores, 2009). The death ligands, TNF and Fas ligand (FasL), are usually found on the surface of immune cells, such as NK and CD8 T cells. In contrast, TRAIL is expressed in a wide range of cells (Wiley et al., 1995).

The most well-known cell death receptor is the Fas receptor. Upon ligand binding, the FasR, organized into clusters, undergo conformational changes, leading to the recruitment of the adaptor protein FADD. Consecutively, pro-caspase-8 is recruited and is auto-catalytically activated (Huang et al., 1999). Activation of caspase-8 by the complex DISC is regulated by the cellular FLICE/caspase-8-like inhibitory protein (cFLIP) (Irmeler et al., 1997). cFLIPs are also recruited to the DISC *via* DED-DED interaction. At the DISC, it blocks caspase-8 activation by competing for binding to FADD. Active caspase-8, under dimer form, leaves the complex DISC and activates the effector caspase-3/7. The cells susceptible to cell death triggered by the extrinsic pathway are called type 1 cells.

In most cases, the extrinsic pathway is insufficient to induce cell death but needs to combine with the intrinsic pathways. Bid cleavage provides a molecular basis for the cross-talk between the two activation pathways of apoptosis (Li et al., 1998). Active caspase-8 cleaves cytosolic inactive Bid into active truncated Bid (tBid), which is translocated into mitochondria. Subsequently, tBid activates Bax and Bak and triggers cytochrome c release.

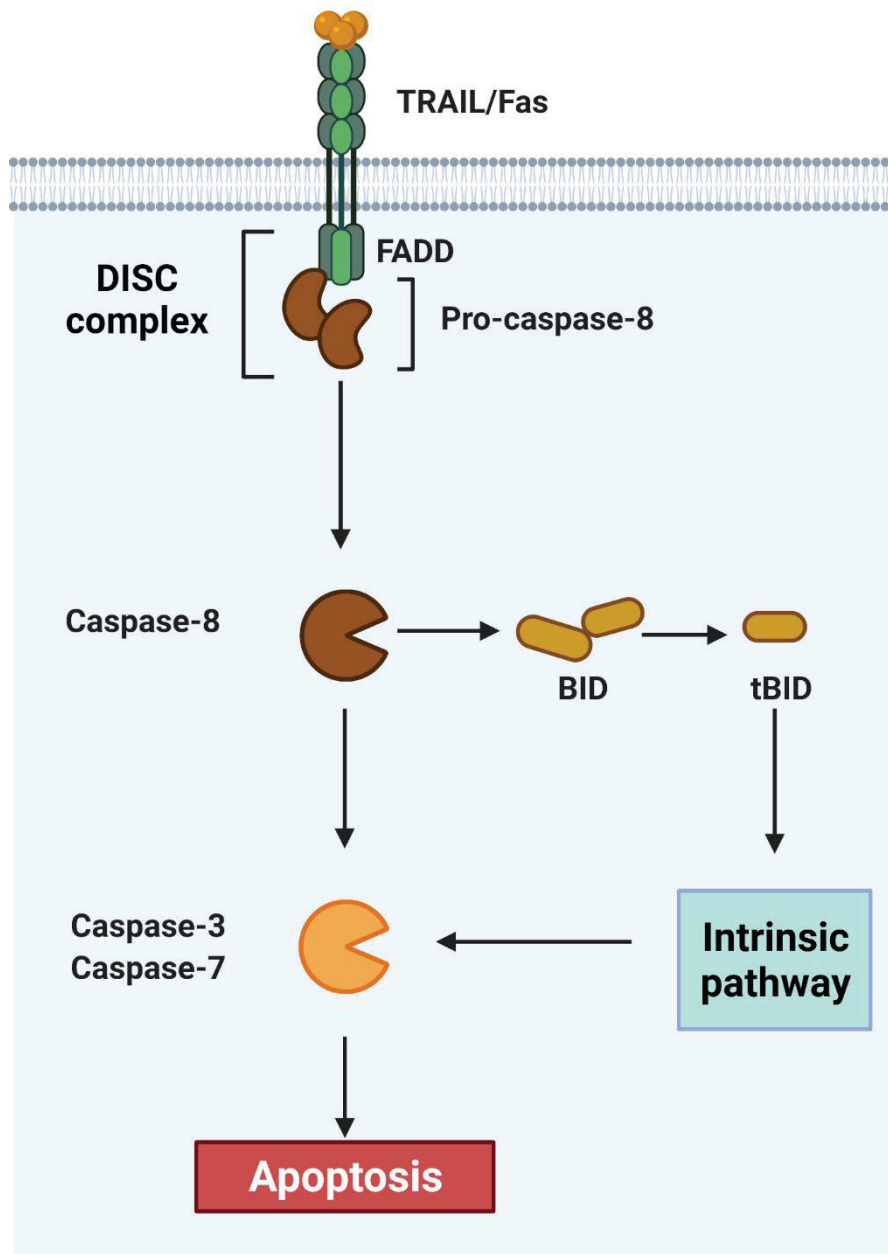


Figure 10. The extrinsic pathway of apoptosis

Upon binding to their ligands, the death receptors TRAILR/FasR are activated and recruit the adaptor protein FADD. Consequently, FADD recruits the inactive form of caspase-8 and forms the DISC complex. After being activated by the DISC complex, caspase-8 activates effectors caspase-3 and -7. Active caspase-8 also cleaves Bid into active truncated Bid (tBid), which links the extrinsic to the intrinsic pathway of apoptosis.

1.3.3.2. Caspase inhibitors

Caspase activity is also controlled by the IAPs. Such control may occur *via* either direct inhibition or degradation through ubiquitination and downstream ubiquitin-targeted proteasome machinery (Joazeiro and Weissman, 2000; Zhang et al., 2019). Intriguingly, XIAP was also shown to be a direct caspase inhibitor, independent of ubiquitin. Indeed, XIAP inhibits the enzymatic activity of both initiator and executor caspases (Eckelman et al., 2006). Following MOMP-induction, other apoptogenic molecules aside from cytochrome c, including Smac/DIABLO, HtrA2/Omi, AIF, and endonuclease G, are also released into the cytosol. In turn, Smac/DIABLO and HtrA2/Omi neutralize the IAPs, resulting in caspase-induced cell remodeling (Du et al., 2000).

1.3.3.3. Regulation through phosphorylation

Both initiator (caspase-8 and -9) and executioner caspases (caspase-3 and -7) are directly regulated by phosphorylation (Parrish et al., 2013; Zamaraev et al., 2017)(Parrish et al., 2013; Zamaraev et al., 2017). Caspase-3 activity has been reported to be inhibited by p38 MAPK through phosphorylation of the highly conserved S150 residue, which is reversed by PP2A (Alvarado-Kristensson et al., 2004; Alvarado-Kristensson and Andersson, 2005). Caspase-9 has the largest subset of reported phosphorylation sites. Most of these sites are located in the large and small subunits but not in the active site (Zamaraev et al., 2017)(Zamaraev et al., 2017). Tyr153 is the only phosphorylation site reported to promote caspase-9 activation, whereas other sites impede auto-processing and block substrate binding (Raina et al., 2005; Allan and Clarke, 2007; Serrano et al., 2017; Serrano and Hardy, 2018). Both inhibitory and activating phosphorylation sites are present in caspase-3, whereas only the former has been described in caspase-7 (Alvarado-Kristensson et al., 2004; Voss et al., 2005; Eron et al., 2017).

2. Non-canonical roles of Bcl-2 proteins: apoptosis and beyond

2.1. Mitochondrial dynamics and metabolism

2.1.1. Mitochondrial dynamics

2.1.1.1. Mitochondrial network

Mitochondria are highly dynamic organelles that undergo continuous cycles of fusion and fission to adapt to cellular context requirements. Fission is the process in which one mitochondrion splits into two daughter mitochondria. Inversely, fusion combines two separated mitochondria into one mitochondrion (Figure 11). The balance between these two processes determines the mitochondrial size, shape, and position, which is referred to as “mitochondrial dynamics”. The mitochondrial dynamics are essential to mitochondria functions in metabolism and other cellular processes (Detmer and Chan, 2007).

Dependent on the ratio between fusion and fission, the result would be a fragmented network with small round-shape mitochondria or a hyper-fused network with elongated, connected mitochondria (Tilokani et al., 2018). The tubular shape is usually referred to as the metabolism-boosting form of mitochondria. Mitochondrial fragmentation is often linked to mitochondrial dysfunction, with some exceptions (Zemirli et al., 2018). Systematic mitochondrial fragmentation is observed before the mitosis, which is essential for their motility, mtDNA inheritance, and quality control (Otera et al., 2013). In a neuron, tiny round mitochondria are the only form observed in the axon, while in the cell body, mitochondria are organized into the tubular network. This small size of axonal mitochondria is critical for its transport and distribution into this narrow cellular compartment (Lewis et al., 2018).

The main actors of these two processes are proteins of the dynamin-like GTPase family. Mitochondrial fission is performed by the Dynamin-related protein 1 (Drp1) and Dynamin 2 (Dnm2). Mitochondrial fusions are mediated by mitofusin-1 and -2 (Mfn1 and 2) and Opa1.

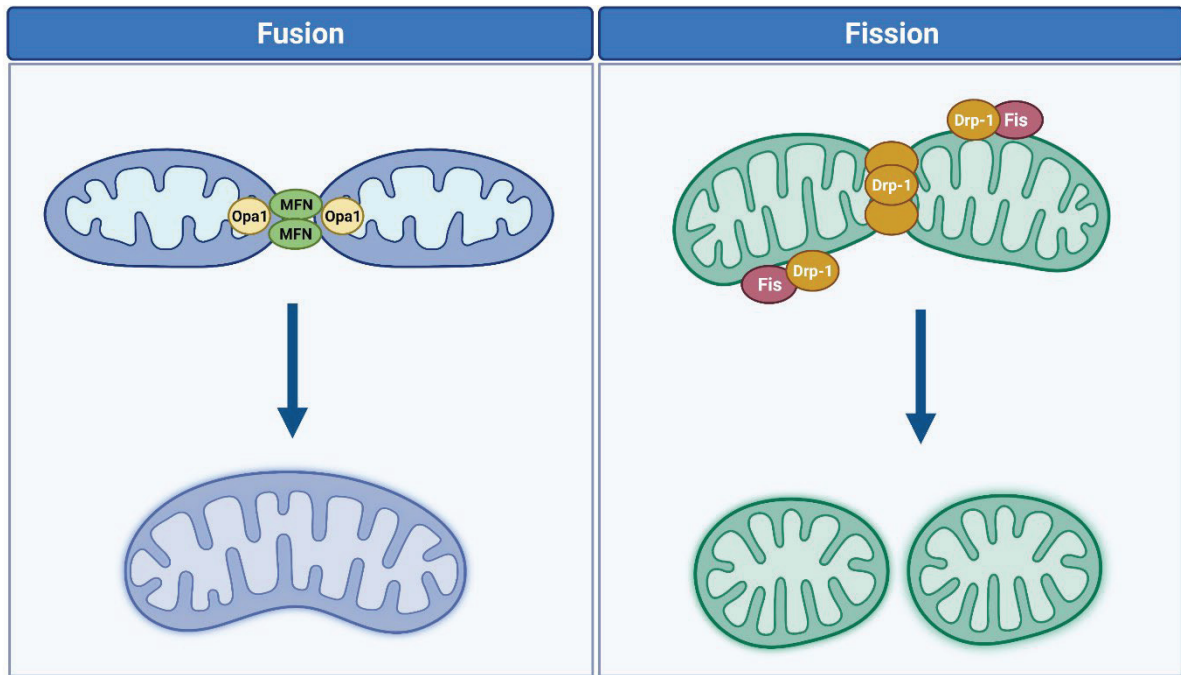


Figure 11. Mitochondrial fusion and fission

Mitochondrial fusion brings two individual mitochondria together to form a new mitochondrion. Fusion is mediated by Mfns at the MOM and Opa1 at the MIM. Mitochondrial fission divides one big mitochondrion into two daughter mitochondria. This process is initiated with the recruitment of cytosolic Drp1 to mitochondria via its receptor and adaptor proteins. In the next step, Drp1 oligomerizes to form a ring-like structure at the future division point and mediates fission.

Mitochondrial fission

Mitochondrial fission initiates with the recruitment of the large GTPase Drp1 to the MOM. It is a cytosolic protein that can be recruited to the mitochondria or peroxisome, where it creates membrane constriction in a GTP-dependent manner (Smirnova et al., 2001).

Drp1 consists of four domains: a GTPase domain at the N-terminus, a middle domain, a variable domain, and a GTPase effector domain (GED). It also contains bundle signaling elements (BSE) and stalk regions that are important for its oligomerization (Okamoto and Shaw, 2005).

For mitochondrial fission, cytosolic Drp1 is recruited to the MOM. Drp1 forms a ring-like structure through oligomerization, which narrows the MOM and sets the constriction site for scission performed by Dnm2 (Rosenbloom et al., 2014).

As Drp1 lacks a pleckstrin homology (PH) domain to bind to the hydrophobic membrane directly, it engages in the MOM *via* its receptors (Otera et al., 2013). In yeast, Drp1 orthologue, Dnm1 is recruited to MOM *via* the membrane-anchored protein mitochondrial fission 1 protein (Fis1) and two receptors, mitochondrial division protein 1 (Mdv1) and CCR4-associated factor 4 (Caf4). In mammals, Drp1 recruitment is mediated by tailed-anchored protein mitochondrial fission factor (Mff) and two receptors mitochondrial dynamics proteins 49 and 51 (MiD49 and MiD51) (Osellame et al., 2016).

Recent findings highlight the role of ER in mitochondria division. Before Drp1 oligomerization, the future scission point of mitochondria is surrounded by ER tubules and is narrowed down to a constriction with a diameter of about 150nm (Friedman et al., 2011). That facilitates the Drp1 ring-like formation. Mitochondria-ER contact sites also mark the location to recruit Drp1 on mitochondria. Besides, ER also provides calcium and lipid that are necessary for membrane remodeling and division.

The cytoskeletal structure also involves mitochondria fission *via* stabilizing the ER-mitochondria contact. Before Drp1 recruitment, actin filament is formed at the mitochondrial constriction (Li et al., 2015). The bridge between mitochondria and ER is formed by nucleating and polymerizing of actin initiated by ER-bound inverted formin 2 (INF2) and mitochondrial spire-type actin nucleation factor 1 (Spire1C) (Korobova et al., 2013; Manor et al., 2015). Moreover, the Drp1 oligomer, which can be assembled at the ER, can be transferred through actin filaments (F-actin) to reach mitochondria to contribute to mitochondrial division (Ji et al., 2017).

Mitochondrial fusion

Mfn1 and 2 are responsible for the fusion of the MOM. Mfns are composed of two TM domains in the middle of the protein sequence that are located between the C-terminal heptad repeat 2 (HR2) and the N-terminal GTPase and heptad repeat 1 (HR1) (Santel and Fuller, 2001; Rojo et al., 2002). Mfns are located at the MOM even at their resting state *via* its two TMD and exposed its GTPase domain and two HR domains in the cytosol.

MOM fusion is initiated with the trans interaction between HR2 and GTPase domains of two Mfn molecules at two opposing mitochondria. GTP binding leads to Mfns conformational changes, bringing two mitochondria near each other. Finally, the fusion of the two MOM is induced by GTP hydrolysis (Tilokani et al., 2018).

MIM fusion occurs consequently to MOM fusion. This process is mediated by Opa1, a large GTPase dynamin-like protein. Opa1 is located in the IMS due to its TM domain (Olichon et al., 2002). The protein structure of Opa1 has not yet been well-defined. Two sites of proteolytic cleavage have been identified in the Opa1 sequence, which leads to the presence of at least five Opa1 fragments (Del Dotto et al., 2018). Moreover, aside from mitochondria fusion, Opa1 is also involved in other processes, such as cristae remodeling (Frezza et al., 2006). With all these complexities and the lack of a 3D structure, the molecular mechanism of Opa1 is still obscure.

2.1.1.2. Bcl-2 protein in the mitochondrial dynamics

2.1.1.2.1. In apoptotic cells

Upon apoptotic stimulus, the mitochondrial network becomes fragmented with the presence of small rounded mitochondria. Expression of a dominant-negative mutant of Drp1 decreases mitochondrial fusion, inhibits cytochrome c release, and delays apoptosis (Frank et al., 2001; Parone et al., 2006). Inhibition of Drp1 impacts selectively the release of cytochrome c but not other apoptogenic agents, such as Smac/Diablo, Omi/HtrA2 (Parone et al., 2006; Estaquier and Arnoult, 2007). Recent findings demonstrate that Drp1 oligomers are created at the ER-mitochondria contact site upon apoptotic stimulus (Prudent et al., 2015b). This platform, mediated by Drp1, enhances the transfer of calcium and lipid to mitochondria, which is important for remodeling cristae junction and liberating cytochrome c. This novel element may explain why inhibition of Drp1 has little impact on the release of other agents during MOMP. It is noteworthy to mention that this Drp1 fission-independent role hardly undermines its fission-inducing role in apoptosis as mitochondrial fragmentation critically decreases metabolic

functions of the organelle and its capacity to buffer calcium and other metabolites, therefore, amplifying also Drp1-induced membrane remodeling.

Interestingly, during the initial stage of apoptosis, Bax translocates and forms punctate foci on the mitochondria. Subsequently, Drp1 and Mfn2 are recruited to these foci of Bax (Karbowski et al., 2002). Bax/Bak stabilized Drp1 docking at mitochondria by enhancing its sumoylation *via* the MAPL (Wasiak et al., 2007; Prudent et al., 2015b). In addition, Bak has been found to interact with Mfn1 and 2: during apoptosis, Bak disassociates with Mfn2 to bind with Mfn1. Strikingly, the capacity of Bak to disassociate with Mfn2 positively regulates the mitochondrial fragment. However, the mechanism through which this interaction regulates mitochondrial fusion/fission is still obscure (Brooks et al., 2007).

2.1.1.2.2. In non-apoptotic cells

Unlike in the apoptotic condition, Bax and Bak are essential to the MOM fusion mediated by Mfn in the healthy cells. Bax/Bak is in charge of Mfn recruitment to foci formation at the fusion sites (Karbowski et al., 2006). This phenomenon is similar to one observed with Drp1 under apoptosis but induces a completely different effect (Karbowski et al., 2002). These two opposite activities may be due to intrinsic conformational changes of pro-apoptotic proteins during apoptosis, which leads to converting the mitochondrial elongation effect of Bax/Bak into Drp1-dependent fission boosting. It is also possible that the modification in Bax/Bak activities on the mitochondrial fusion/fission reflects the differences in their binding partner under two cellular contexts.

Among anti-apoptotic proteins, Bcl-xL has been found to implicate mitochondrial dynamics. In cortical neurons, Bcl-xL promotes both fusion and fission processes but shifts the balance between two processes toward fusion. Consequently, overexpression of Bcl-xL results in more elongated mitochondria in the axons (Berman et al., 2009a).

2.1.1.2.3. How do Bcl-2 proteins impact the mitochondrial network?

Bcl-2 proteins, including Bax, Bak, Bcl-xL, and Bcl-2, are shown to interact with Mfn *via* co-immunoprecipitation (Delivani et al., 2006; Karbowski et al., 2006). Regarding Drp1, hitherto, only Bcl-xL has been demonstrated to bind to this protein directly and is thought to enhance the activity of the GTPase domain of Drp1 (Li et al., 2008a). These observations suggest that Bcl-2 proteins potentially modulate the machinery of fusion and fission through direct regulating activities of these proteins. However, the molecular mechanism and, sometimes, the biological consequences of these interactions are unknown. Noteworthy, in some cases, Bcl-2

proteins may be served as a protein adaptor to facilitate the interaction or biochemical reaction between proteins. For instance, during the initial step of apoptosis, Bax recruits Drp1 and MAPL to mitochondria foci to promote the sumoylation of Drp1 (Prudent et al., 2015b).

Indirect regulation of Bcl-2 proteins to mitochondrial dynamics has not been described. Notwithstanding, since Bcl-2 proteins regulate calcium signaling and other metabolite transfer, which also mediates activities of fusion and fission, this possibility is highly imaginable. For instance, Drp1 activity is regulated by post-translational phosphorylation. Phosphorylation at Ser616 of Drp1 promotes fission. Inversely, phosphorylation at Ser637 deactivates Drp1 and increases its shuttling back to the cytosol (Wang et al., 2012b; Perdiz et al., 2017). Phosphorylation of Ser616 by Ca^{2+} /calmodulin-dependent kinase II (CaMKII) and dephosphorylation of Ser637 by calcineurin (CaN) are both calcium-dependent (Cribbs and Strack, 2007; Xu et al., 2016). Thus, it would be interesting to see if Bcl-2 calcium regulation also modulates the activity of Drp1.

2.1.2. Mitochondrial metabolism

Aside from regulating the mitochondrial metabolism *via* impacting the mitochondrial morphology and network as described in the previous part and *via* Ca^{2+} regulation that will mention in the next part, Bcl-2 proteins can also regulate mitochondrial metabolism by directly regulating the respiratory complexes.

Mcl-1 is involved in the electron transport chain (ETC) complex assembly. Mcl-1 deletion leads to mitochondrial morphological defects, including fragmented network and abnormal cristae. A cleavage signal at the N-terminus targets the truncated isoform to the mitochondrial matrix, where it normally couples mitochondrial fusion to respiration (Perciavalle et al., 2012). This isoform promotes the assembly of large respiratory complexes of complexes I, III, and IV situated in the mitochondrial cristae. Furthermore, it also enhances the oligomerization of ATP synthase into a supramolecular complex (Figure 12A). The assembly of these large complexes promotes mitochondrial ATP synthesis (Thomas et al., 2008; Lapuente-Brun et al., 2013).

Bcl-xL has also been found in the MIM. There, it interacts with ATP synthase and promotes ATP production *via* diminishing the proton leak (Figure 12B) (Alavian et al., 2011). The role of this pool of Bcl-xL is thought to be essential for synapse formation and function in the hippocampal neurons.

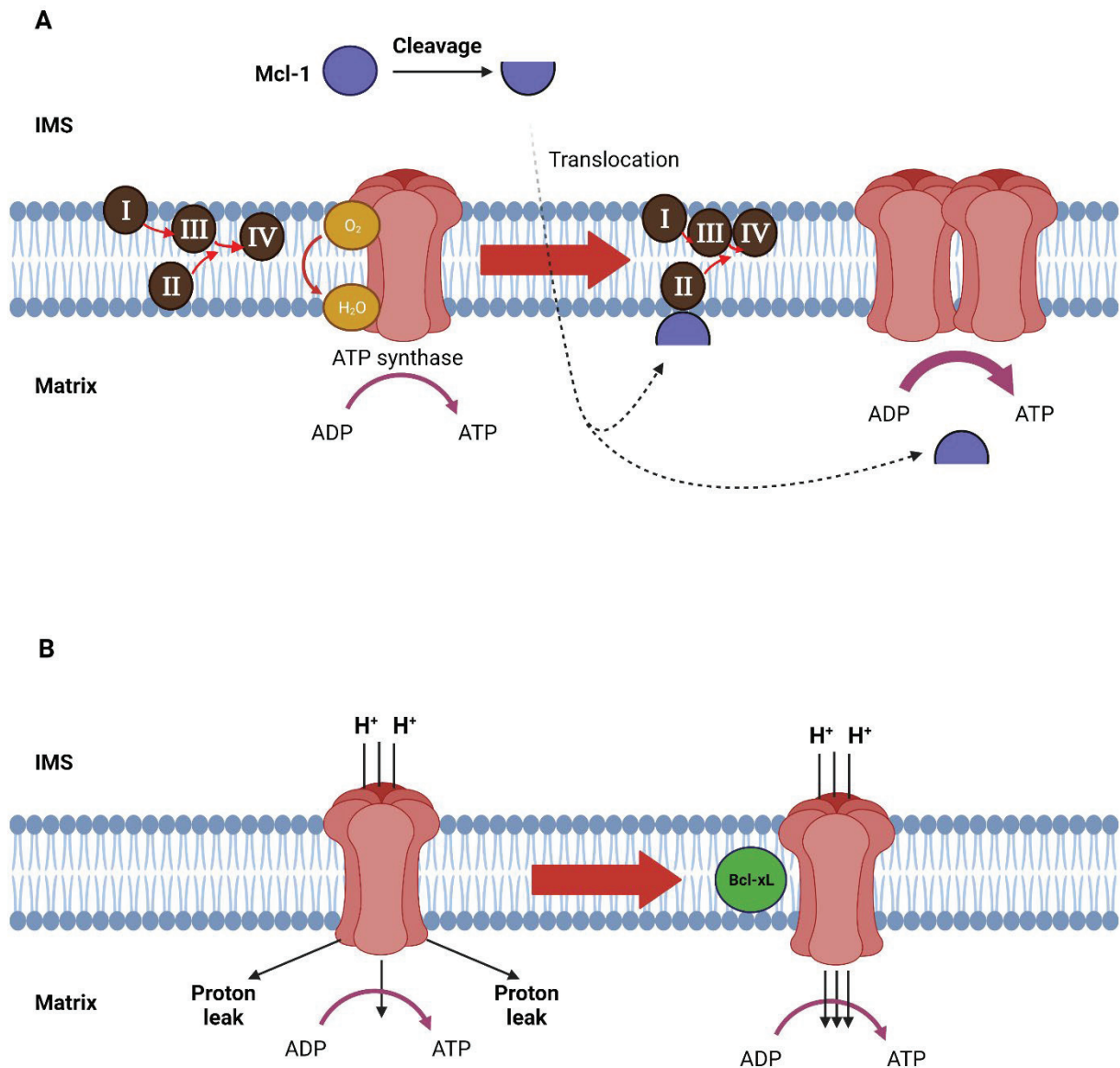


Figure 12. Implication of Bcl-2 proteins in mitochondrial metabolism

(A) Mcl-1 is proteolytically cleaved to form ΔN -Mcl-1 isoform, which is then translocated from IMS into the mitochondrial matrix. There, ΔN -Mcl-1 promotes ATP production by stabilizing the macro-complex of respiratory complex I, III, and IV and enhancing the activity of ATP synthase. (B) Bcl-xL interacts with ATP synthase in the IMM and prevents the proton leak through this complex, therefore optimizing ATP production. Figure adapted from (Giménez-Cassina and Danial, 2015).

2.2. Calcium regulation

2.2.1. Short words about cellular calcium

Calcium plays an important role in a plethora of cellular physiological processes and also in pathological progression and resistance, including cancer. The majority of intracellular Ca^{2+} is stored in the ER lumen (Raffaello et al., 2016). Thus, this organelle is important to Ca^{2+} regulation. Ca^{2+} is pumped into ER through the Sarco/endoplasmic reticulum ATPase (SERCA). The efflux of Ca^{2+} from ER is controlled by the IP_3R . Ca^{2+} efflux *via* IP_3R is organized into three forms of signal adapting to cell requirements: a prolonged elevation of Ca^{2+} flux or a single transient Ca^{2+} . The storage of Ca^{2+} in the ER can be replenished by the extracellular environment through a mechanism termed store-operated Ca^{2+} entry (SOCE), which allows Ca^{2+} to flow directly from outside into ER without disturbing cytosol. This process involves the ER Ca^{2+} sensors Stim and the plasma membrane Ca^{2+} -influx channels Orai (Carreras-Sureda et al., 2018).

The mitochondrion is an organelle with an important Ca^{2+} buffering capacity. Moreover, Ca^{2+} is essential to the normal functions of mitochondria in metabolism and apoptosis regulation (Duchen, 2000). Communication between ER and mitochondria is performed at a platform called mitochondria-ER contact sites (MERCs). MERCs are dynamic contact regions with a distance of 10-30nm between two organelles, which compose a platform of Ca^{2+} transfer and metabolite exchange. Ca^{2+} transport proteins are present on this platform, including IP_3Rs and VDACs, which are mitochondrial Ca^{2+} uptake channels (Patergnani et al., 2011). The Ca^{2+} exchange is strictly regulated as it regulates the mitochondrial oxidative phosphorylation (OXPHOS) on the one hand but also triggers apoptosis on the other hand (Giorgi et al., 2012). As potential apoptosis regulators, actions of Bcl-2 protein also reach up to ER, where they can execute their functions in a remote way. Finally, these interactions also have outcomes on the physiological processes governed by Ca^{2+} signals.

2.2.2. Bcl-2 protein regulating ER Ca^{2+}

Multiple Bcl-2 proteins are found at the ER; this pool of Bcl-2 proteins also contributes to Bcl-2 apoptosis-related effect. The first simple model proposed that Bcl-2 anti-apoptotic proteins suppress Ca^{2+} release from ER, therefore preventing mitochondrial Ca^{2+} overload and Ca^{2+} -induced apoptosis. Hitherto, it is clear that the Ca^{2+} regulation mechanism by Bcl-2 is a lot more complex *via* modulating activities of multiple components of the Ca^{2+} -regulating system (Figure 13).

Accordingly, Bcl-2 anti-apoptotic proteins regulate this Ca^{2+} communication between two organelles. They sensitize IP_3R to a low concentration of IP_3 , allowing Ca^{2+} transfer to mitochondria. Reversely, they inhibit IP_3R -mediated Ca^{2+} release during pro-apoptotic signaling where IP_3 concentration is elevated. Bcl-2 anti-apoptotic proteins also promote or inhibit VDAC-mediated mitochondrial calcium uptake, correspondingly.

Furthermore, Bcl-2 regulates ER Ca^{2+} storage by promoting IP_3R -related Ca^{2+} and interacting with SERCA. Interaction between Bcl-2 and SERCA is controversial: it can be either inhibitory or excitatory depending on the context.

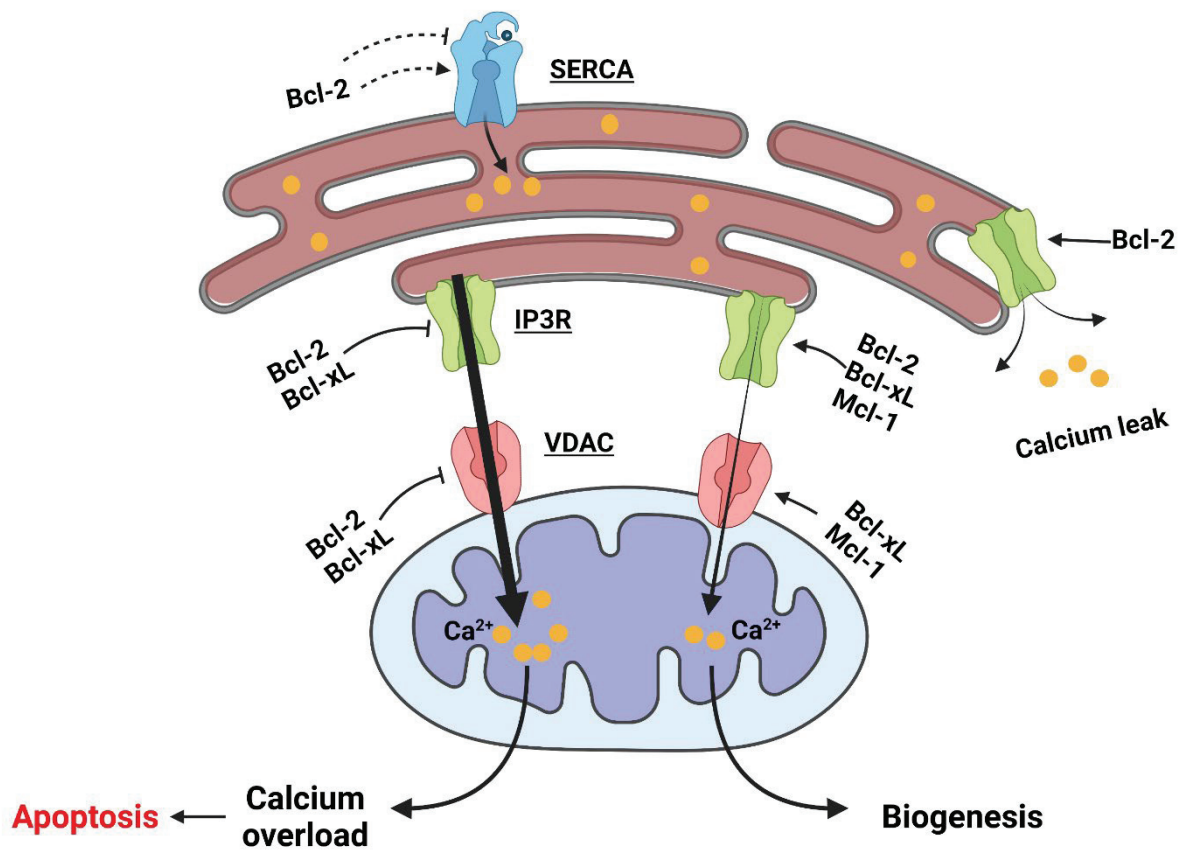


Figure 13. Modulation of ER and mitochondrial Ca^{2+} by anti-apoptotic proteins

Ca^{2+} between ER and mitochondria defines cell fate. This flux is mediated by IP₃R at the ER, VDAC at the MOM, and MCU at the MIM (do not show). In the healthy cell, Ca^{2+} oscillation promotes mitochondrial biogenesis (green arrow). In contrast, abundant Ca^{2+} flux to mitochondria leads to Ca^{2+} overload and apoptosis (red arrow).

2.2.2.1. *Via regulating ER Ca²⁺ pool*

Bcl-2 proteins regulate the steady-state ER Ca²⁺ levels, which can impact the capacity to release apoptotic Ca²⁺ waves. The ER Ca²⁺ steady-state is defined by two independent tendencies: Ca²⁺ uptake *via* SERCA and Ca²⁺ release/leak *via* IP₃R. Hitherto, pieces of evidence have demonstrated the implication of Bcl-2 proteins in these two steps.

The first hint of Bcl-2 proteins regulating ER Ca²⁺ content was observed in the Bcl-2 overexpressed cells. When the anti-apoptotic Bcl-2 is over-expressed, the free Ca²⁺ in ER lumen is decreased. That is the consequence of the increased ER²⁺ leak and is independent of mitochondrial Ca²⁺ uptake (Foyouzi-Youssefi et al., 2000; Pinton et al., 2000). This action is considered a powerful anti-apoptotic activity in a preventative way, as it limits the releasable quantity of Ca²⁺ in response to apoptotic stimuli.

The Bcl-2-induced ER Ca²⁺ leak is potentially regulated *via* interactions between anti-apoptotic and pro-apoptotic proteins. The first evidence for this model was described by Korsmeyer team. This study shows that phosphorylation of Bcl-2 at an unstructured loop between its BH4 and BH3 motif diminishes its activity on ER Ca²⁺ leak. Interesting, phosphorylation at this point on Bcl-2 also impairs its interaction with Bax and BH3-only proteins. This model was further supported by a later study published by this team. In this study, Oakes and colleagues found that KO of *bax* and *bak* also decreased the ER Ca²⁺ store as induced by Bcl-2 overexpression. Strikingly, loss of Bax and Bak enhances the interaction between Bcl-2 and IP₃R at the ER, which leads to a hyper-phosphorylation state of IP₃R by protein kinase A (PKA). These observations support the model in which Bcl-2 directly regulates IP₃R-related Ca²⁺ leak *via* phosphorylation, and this function is modulated by Bcl-2 anti-apoptotic proteins *via* a competitive inhibition mechanism (Oakes et al., 2005).

As bearing no kinase domain, Bcl-2 is likely to function as a protein recruiting platform to regulate IP₃R functions. A recent study demonstrated that Bcl-2 recruits the Ca²⁺-activated phosphatase CaN and dopamine and cAMP-regulated phosphoprotein (DARPP-32), a CaN-regulated inhibitor of protein phosphatase 1 (PP1). That brings these two proteins in proximity with IP₃R *via* interaction with Bcl-2. The Ca²⁺ release elevation is detected by CaN, which then dephosphorylates DARPP-32, so activates PP1. Consecutively, PP1 dephosphorylates IP₃R, therefore decreasing Ca²⁺ release (Chang et al., 2014).

Bcl-2 activities on ER are also *via* interacting with Bax inhibitor 1 (BI-1), a protein regulating ER Ca²⁺ through sensitizing the IP₃R, but also *via* oligomerizing and acting as an H⁺/Ca²⁺

exchanger channel. Bcl-2 directly binds to BI-1 *via* its BH4 motif. This interaction promotes BI-1 oligomerization and Ca²⁺ exportation (Xu et al., 2008). The presence of BI-1 is important for the Bcl-2 mediated ER Ca²⁺ lowering.

Bcl-2 proteins also regulate ER Ca²⁺ *via* SERCA function modulation. Bcl-2 can interact directly and indirectly with all three isoforms of SERCA. However, the effect of this interaction is controversial in the literature. Bcl-2 has been shown to interact directly with the SERCA1 and inactivate it by completely inhibiting the folding of skeletal muscle cell SERCA1 (Dremina et al., 2004). In the oocyte of *Xenopus*, Bcl-2 over-expression induced SERCA2 upregulation and preserved SERCA activity in the presence of SERCA inhibitor thapsigargin (Kobrinisky and Kirchberger, 2001). One possible hypothesis explaining these different effects of Bcl-2 on SERCA is that it depends on the isoform of SERCA. Three SERCA isoforms are expressed in a tissue-specific: SERCA1 is highly dominant in the skeletal muscle type II, SERCA2a in the skeletal muscle type I, smooth muscles, and cardiac myocytes, SERCA2b is widely expressed, and SERCA3 is usually expressed in specified non-muscle cells in combination with SERCA2b (East, 2000). Even though SERCA1 et SERCA2a share high similarities in the structure and Ca²⁺ affinity, it is not possible that Bcl-2 impacts differently on different isoforms. The divergence of Bcl-2 – SERCA interaction would be ideal for Bcl-2 anti-apoptotic roles. SERCA inhibition lowers the ER Ca²⁺ store, preventing high apoptotic Ca²⁺ release waves. However, complete SERCA inhibition, leading to the ER Ca²⁺ depletion, triggers the ER stress and can result in apoptosis.

2.2.2.2. *Via inhibiting proapoptotic Ca²⁺ waves from ER*

The protein Bcl-2 has been found to directly inhibit IP₃R-mediated Ca²⁺ release, independent of its activity on ER Ca²⁺ content (Hanson et al., 2008). This interaction, *via* the regulatory and coupling domain of the IP₃R, inhibits exaggerated Ca²⁺ release by IP₃R and blocks the Ca²⁺-induced apoptosis (Rong et al., 2009). BH4 motif of Bcl-2 is sufficient for its interaction with IP₃R and Ca²⁺-related anti-apoptotic function (Rong et al., 2009). BH4 motif of Bcl-2 binds to all three isoforms of IP₃R (Monaco et al., 2012). However, its anti-apoptotic activity is likely mediated *via* its interaction with IP₃R2 (Akl et al., 2013).

Even though multiple Bcl-2 anti-apoptotic proteins present a BH4 motif, interaction with the modulatory domain of IP₃R is not likely to be a common feature between these proteins. For example, it was demonstrated that the positive charged residue Lys17 of the Bcl-2 BH4 motif is critical for its interaction with IP₃R. In Bcl-xL, this residue corresponds to Asp11, which explains the low interaction between the BH4 motif of Bcl-xL with the modulatory domain

(Monaco et al., 2012). Actually, a recent study from the same team showed that the full-length Bcl-xL was required for binding to the modulatory domain of IP₃R. This observation is in line with our previous result about Nrz-IP₃R interaction. Indeed, the BH4 motif of the protein Nrz is insufficient for its interaction with the binding domain of IP₃R and its inhibitory effect on this calcium channel (Bonneau et al., 2014). Although full-length Bcl-xL has a lower affinity with IP₃R than Bcl-2, the interaction between Bcl-xL and IP₃R is sufficient to inhibit the pro-apoptotic Ca²⁺ release (Rosa et al., 2022). This study aligns with the one of Foskett team, in which he and his colleagues proposed that Bcl-xL has a biphasic effect on IP₃R. At high concentrations, Bcl-xL inhibits the Ca²⁺ *via* IP₃R (Yang et al., 2016).

2.2.2.3. *Via promoting Ca²⁺ oscillations through IP₃R*

In contrast to the pro-apoptotic elevated Ca²⁺, the regular small spikes of Ca²⁺ release, or called Ca²⁺ oscillations, are pro-survival. They provide the mitochondria with the appropriate quantity of Ca²⁺ to boost ATP synthesis. Indeed, Ca²⁺ is necessary for three Ca²⁺-sensitive enzymes who implicate in the tricarboxylic acid cycle (Kreb's cycle), including pyruvate dehydrogenase, isocitrate dehydrogenase, and α -ketoglutarate dehydrogenase (Hajnóczky et al., 1995).

Besides the central modulatory domain of the IP₃R, Bcl-2 anti-apoptotic proteins, including Bcl-xL, Bcl-2, and Mcl-1, also interact with the C-terminal region of the IP₃R (White et al., 2005; Eckenrode et al., 2010). The C-terminal domain of IP₃R is composed of six TM helices, which are responsible for membrane anchoring and contribute to the ion-permeable pore (Foskett et al., 2007). Binding Bcl-2 anti-apoptotic proteins to the C-terminus of IP₃R sensitizes the IP₃R with low IP₃ concentration and induces Ca²⁺ oscillations (White et al., 2005; Eckenrode et al., 2010). In the protein Bcl-xL, this interaction is likely to occur between the BH3-like motif in the C-terminus of IP₃R with the hydrophobic groove of Bcl-xL (Foskett et al., 2009). This interaction can be disrupted by the presence of Bax or BH3-only protein, tBid.

2.2.3. Bcl-2 protein regulating mitochondrial Ca²⁺

The communication *via* Ca²⁺ between the ER and mitochondria is essential to regulate mitochondrial metabolism and apoptosis signaling. Interestingly, IP₃R has been found to link to VDAC, the mitochondrial uptake channel *via* Glucose-regulated protein 75 (GRP75), which forms a straight platform for Ca²⁺ transfer between two organelles (Szabadkai et al., 2006).

Bcl-2 anti-apoptotic proteins have been found to regulate the activity of VDAC. The effect of this interaction is likely context-dependent.

Using Bcl-xL KO fibroblasts, Huang and coworkers showed that mitochondrial Ca^{2+} uptake is compromised on Bcl-xL lacking mitochondria. They further demonstrated that this effect is mediated by the interaction between Bcl-xL with VDAC 1 and 3 (Huang et al., 2013). A later study suggests that Mcl-1 shares the same characteristics. In the context of lung cancer, Mcl-1 also interacts with VDAC 1 and 3 to promote mitochondrial Ca^{2+} uptake that enhances the migration *via* ROS production increase (Huang et al., 2014).

In contrast, Bcl-2 and Bcl-xL inhibit VDAC activity in response to apoptotic stimuli (Shimizu et al., 2000). This interaction is mediated by the BH4 motif of Bcl-2 and Bcl-xL proteins. However, in this study, the authors did not decipher if this phenomenon was related to calcium uptake or the cytochrome c release role of VDAC. A further study by Monaco and his colleagues suggests that the BH4 motif of Bcl-xL indeed interacts with VDAC1 to inhibit apoptotic Ca^{2+} wave (Monaco et al., 2015).

2.3. Cytoskeletal dynamics and migration

Cytosolic Ca^{2+} regulates the reorganization of the cytoskeleton, especially controlling the activity of actomyosin and the microtubule network. In addition, Ca^{2+} signals the reorganization of the focal adhesion (FA) complex *via* focal adhesion kinases (FAKs), which contribute to cell movements and migration (Tsai et al., 2015). As regulators of cytosolic Ca^{2+} due to their actions on ER and mitochondria, Bcl-2 proteins have been found to implicate in the cytoskeletal remodeling and cellular migration (Prudent et al., 2015).

A very specific example is the developmental role of Nrz, a zebrafish orthologue of Nrh. During the early stage of development, Nrz is highly expressed in the yolk syncytial layer (YSL). At the ER, this protein binds to the IP_3 -binding domain of IP_3R . This interaction inhibits the binding of IP_3 to its receptor, therefore regulating the Ca^{2+} signals during epiboly (Bonneau et al., 2014). Knockdown of Nrz induced a remarked increase of cytosolic Ca^{2+} in the YSL, which led to the perturbation of the microtubule and F-actin network. Increased Ca^{2+} level augments the phosphorylation of the myosin light chain, which leads to premature formation of the actin-myosin ring and hypercontraction in the margin, separating the blastomeres from the yolk cell (Popgeorgiev et al., 2011). Altogether, Nrz presence is critical to maintaining the normal development during epiboly of zebrafish.

Bcl-2 proteins also control cell migration and invasion by regulating the production of mitochondrial reactive oxygen species (ROS). In healthy cells, overexpression of anti-apoptotic proteins promotes ROS production, while pro-apoptotic proteins oppose this effect

(Kowaltowski et al., 2004; Kim et al., 2012). The quantity of ROS increased by anti-apoptotic protein overexpression is not sufficient to trigger cell death *via* oxidative stress but rather supports cell migration. Mitochondrial ROS activates various signaling pathways and molecules involved in cell migration and invasion, including phosphoinositide 3-kinase (PI3K), MAPK, matrix metalloproteinase (MMP), etc. (Tochhawng et al., 2013).

The activity of Bcl-xL on cell migration is likely independent of its anti-apoptotic function (Choi et al., 2016). Overexpression of Bcl-xL is correlated with increased cell migration and metastasis by enhancing the prosurvival cytokine production and decreasing interaction with the extracellular matrix (Fernández et al., 2000). In a pancreatic tumor model, Bcl-xL promotes the epithelial-to-mesenchymal transition and cell migration through increasing the Transforming growth factor β (TGF β) signaling (Choi et al., 2016). In glioma cells, Bcl-xL overexpression increases TGF β and MMP-2 and enhances cell invasion (Weiler et al., 2006). The protein Bcl-2 also upregulates MMP, especially MMP-2 and MMP-9 (Wick et al., 1998).

This plethora of molecules induced by Bcl-2 proteins may be mediated by transcription factor upregulation. Both Bcl-2 and Bcl-xL overexpression results in increased activation of nuclear factor kappa B (NF- κ B), which then upregulates target genes involved in cell migration (Mortenson et al., 2003; Gabellini et al., 2008). Notably, it has been reported that NF- κ B can be activated by intracellular Ca^{2+} increase (Pahl and Baeuerle, 1996). Another transcription factor is also regulated by Ca^{2+} level is the activator protein 1 (AP-1). When intracellular Ca^{2+} concentration is elevated, CaMK is activated and promotes the formation of the AP-1 transcriptional complex (Schaefer et al., 1998). Bcl-xL regulates ROS production through its interaction with VDAC1. A recent study from our team demonstrated that ROS production is important for Bcl-xL promoting cell migration (Bessou et al., 2020). These observations may propose a molecular mechanism explaining how Bcl-2 anti-apoptotic protein impacts multiple signaling pathways to enhance migration.

Furthermore, some studies showed that Bcl-2 proteins interact directly with different components of the cytoskeletal network. Bcl-2 binds to gelsolin, dampens the severing activity mediated by gelsolin, and enhances actin polymerization (Ke et al., 2010). In vitro experiment also showed the interactions between Bcl-2 proteins with microtubule and its polymerization. Bcl-2, Bad, and Bid inhibit microtubule polymerization and assembly, while Bax and Bak promote this process (Knipling and Wolff, 2006). Nonetheless, the biological significance of these interactions remains to be determined.

3. Development of the central nervous system

3.1. Short summary about the central nervous system

3.1.1. Organization of the central nervous system

The nervous system is the organism's controlling central: it receives sensory information from the environment, processes them, and generates adapting responses and behaviors. Compared to invertebrates, the nervous system of vertebrates is more complex and organized into specialized centers. In mammals, the nervous system is composed of the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS includes the brain and the spinal cord. The PNS comprises the sensory neurons and motoneuron nerves, which innervate the skeletal muscles (somatic nervous system) or the smooth muscles, myocytes, and glands (autonomic nervous system).

At the cellular level, the nervous system consists of neurons and glial cells, including microglia, astrocytes, and oligodendrocyte. Neurons are bodily messenger which generates electrical signals to transmit transformation and modulate functions of other cells such as muscle and gland cells. Glial cells do not take part in the electric signal transmission but perform instead supporting roles. Glial cells support and promote the connection between neurons and regulate their activity. Microglia, sharing many properties with the macrophage, is a scavenger cell that removes cellular debris from dead cells. Oligodendrocyte, strictly staying in the CNS, generates the myelin, a lipid-rich structure wrapping around a neuron structure called the axon. In the PNS, this process is performed by Schwann cells. Microglial cells maintain the appropriate chemical environment for neuronal signaling (Purves et al., 2018).

The CNS is organized into two distinct regions: one rich in the neuronal cell bodies and the other rich in axons. The former is named gray matter, and the latter is white matter. The gray matter can be arranged in two ways. A rough cluster of neurons with similar functions and connections form a nucleus. Otherwise, a cortex, for example, cerebral cortices, is a more organized sheet-like arrangement of neurons.

At the limit of this thesis, I will focus on the neuron and its development.

3.1.2. The cellular structure of neurons

Neurons are the most knowingly polarized cells, with different processes extending from the cell center. A neuron is commonly composed of the cell body, dendrites, and axon (Figure 14) (Raine, 1999). The cell body, or soma, is the round part of neurons containing the nucleus and

surrounding cytoplasm. Dendrites are short and highly branched processes extending from the cell body that receive information from other neurons or the other cells (Goldberg, 2003). The axon is a long process, possibly up to one-meter length, specialized in sending information from its host neuron to other cells. A neuron possesses only one axon; some do not have one.

At the point where the axon of one neuron approaches the dendrite of the other neuron, it forms the synapse, a specialized structure that allows communication between neurons. There are two well-known types of synapses: electrical and chemical, the latter being the most abundant in the CNS. In electrical synapses, the presynaptic physically connects with the postsynaptic membrane through the protein connexon, forming a tightly connected zone called gap junctions. The ion flux is moved directly into the postsynaptic neuron through pores at gap junctions. Otherwise, the chemical synapse does not develop physical contact: the presynaptic terminal separates from the postsynaptic membrane by the synaptic cleft. The communication between two neurons is performed by the secretion of a neurotransmitter in the synaptic cleft (Purves et al., 2018) (Figure 14).

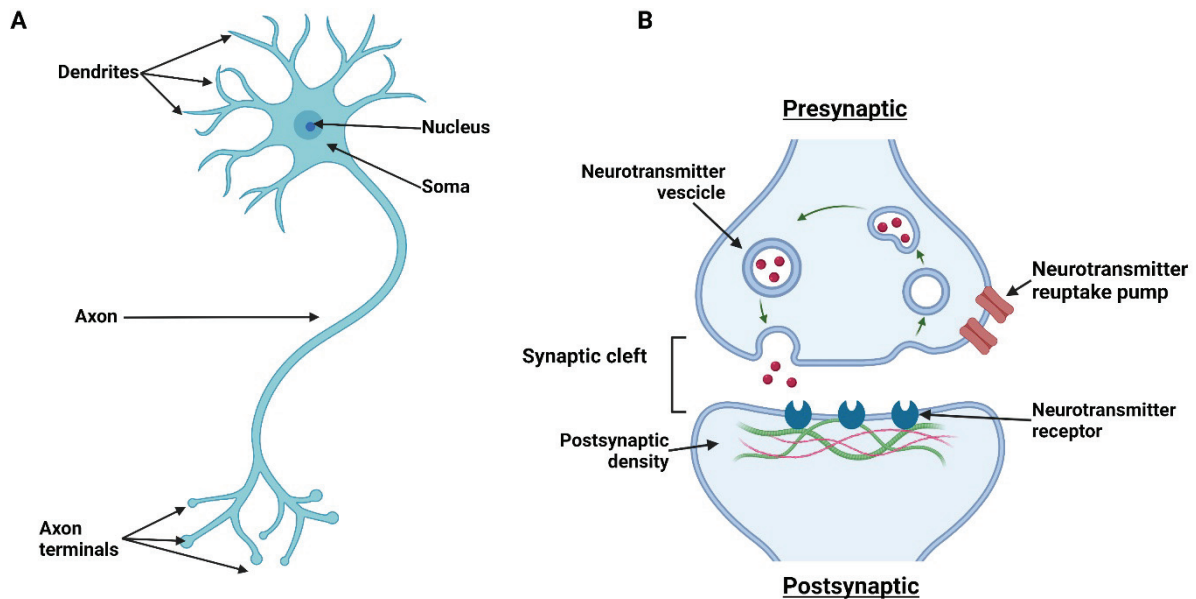


Figure 14. Neuron structure and synapse

(A) Simple schematic representative of the neuron. A neuron is composed of the soma and projections. The nucleus situates in the soma. Among the projections, one develops into an axon, the others into dendrites. The terminals of the axon are branched and develop into synapses, the specialized intercellular communication structure. **(B)** Schematic representative of the chemical synapse. A chemical synapse is composed of a presynaptic (on the axon of a neuron) and a postsynaptic part (on the dendrite of another neuron). In the presynaptic part, neurotransmitters are stored in specialized vesicles. In response to appropriate stimuli, neurotransmitters are released into the synaptic cleft and bind to their receptors which are fixed on the post-synaptic membrane. Neurotransmitters are subsequently re-pumped back to the presynaptic, and the vesicles are recycled.

3.2. Development of the CNS

3.2.1. At organ level

The CNS begins to appear in the early stages of embryonic development. The first significant event is the formation of the neural tube from the neural plate during primary and secondary neurulation. During primary neurulation, a portion of the dorsal ectoderm is specified into the neural plate. Neural plate cells can be distinguished from surrounding non-neural cells by their elongated morphology. Shortly after its establishment, the neural plate border thickens and rises up to create a U-shape invagination. The two lateral sides of the neural groove continue to bend towards each other until their edges meet and merge to form the neural tube. Secondary neurulation then begins at the caudal end. In this latter process, which takes place in chicken and some mammalian embryos, mesenchymal cells gather to form the medullary cord under the ectoderm layer. In the next step, the medullary cord is reshaped to create a hollow cavity (Smith and Schoenwolf, 1997; Gilbert, 2000).

From a simple straight structure at the beginning, the anterior part of the neural tube expands to form three primary vesicles: prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain). These latter vesicles continue to subdivide and ultimately form the different regions of the brain (Smith and Schoenwolf, 1997; Stiles and Jernigan, 2010). Concurrently, neural progenitors, derived from the neuroepithelial cells of the ectoderm, differentiate into neurons and glial cells (Götz and Huttner, 2005; Paridaen and Huttner, 2014). Synchronously with neural differentiation, post-mitotic neurons migrate to attain their right place so that they can interact and form appropriate connections with their targets. Migration requires performing correctly so that the developing neurons can reach their right place at the right time to create precise connections for their proper function. Migration of neurons occurs in both the CNS and PNS development. In the brain cortex, newborn cells migrate outward from the luminal germinal neuroepithelium to form layers at the marginal zone (Ayala et al., 2007). The most recently born neurons migrate through the previous cell layers under the guidance of glial cells to give rise to the more superficial regions.

3.2.2. At cellular level

3.2.2.1. Neuronal polarization

To obtain its asymmetric morphology, the neuron undergoes a process of polarization. A basic model of neural polarity was suggested by Banker and colleagues, which was established from their work on the cultured hippocampal neuron. A neuron undergoes a five-stage morphological

change process in vitro (Figure 15) (Dotti et al., 1988). Newly plated neurons possess tiny extensions like filopodia. From these filopodium, develop the small equivalent neurites. The next critical step is to define the axon among the extensions: one neurite grows rapidly to become the axon. Then, the remaining small neurites grow and branch into dendrites. Lastly, synapses can be formed between mature axons and dendrites.

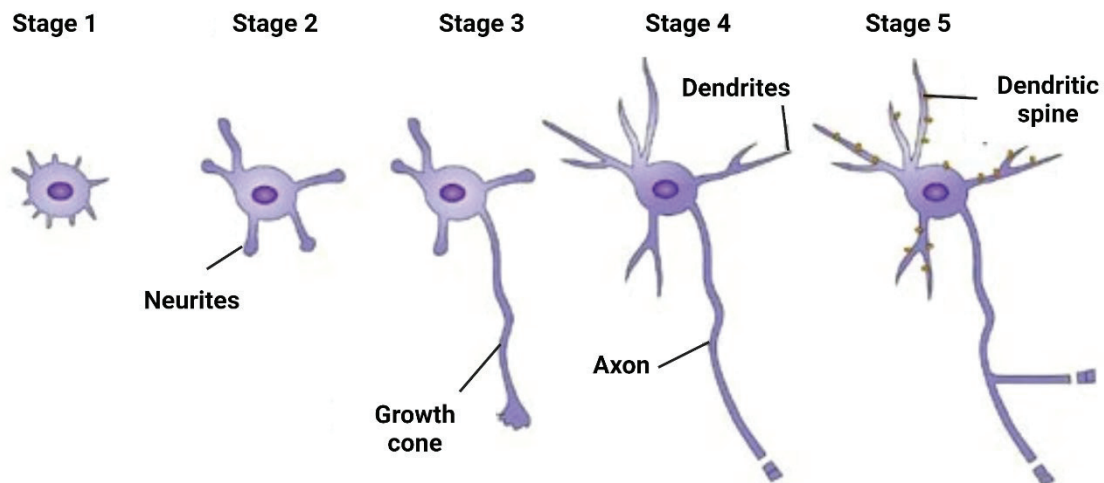


Figure 15. Polarization of the neuron in vitro via a multi-step process

Firstly, small filopodia develop into short neurites. One neurite among them will be polarized into an axon that spread significantly more important than the dendrites. Axonal elongation is directed by the growth cone formed at the distal tip of the axon. The remaining neurites are then highly branched and develop into dendrites. At the late stage, connexions are formed between the axon and the dendrite via a specialized structure called the synapse. From (Arimura and Kaibuchi, 2007)

In vivo, neural polarization processes are different depending on the brain region. Some, such as vertebrate retinal ganglion cells, already have a bipolar morphology at the beginning. The cortical neurons, otherwise, acquire their polarity during migration. These neurons are generated in the ventricular zone (VZ) of the brain and migrate through the subventricular zone towards the intermediate zone (IZ). During this process, immature neurons develop into multipolar cells (MP) with multiple minor neurites. One among these extensions grows rapidly to become a trailing process; another develops into a leading process. The remaining neurites are retracted: the MP transforms into the BP cell in the IZ. The BP neurons complete the polarization and migrate toward the cortical plate to form cortex layers. This process is well-

established in the literature and serves as a polarization model of neurons in vivo (Takano et al., 2015).

3.2.2.2. Axon guidance and branching

3.2.2.2.1. Characteristics of axon guidance and branching

Axon must navigate through a relatively long distance to reach its target destination. During its exploration, the axon is guided by a highly motile structure called the axon growth cone. It is a specialized transient structure at the tip of the developing axons, composed of a sheet-like expansion named lamellipodium. From the lamellipodium, various fine extensions called filopodia can be developed. These structures function as the sensor arms exploring the environment cues to help the growth cone decide which direction to come (Dent et al., 2011). However, the axon growth cone does not establish a direct connection with the axon's target. The formation of connection with neuron targets is performed by collateral axonal branches (Szebenyi et al., 1998).

To form a complex neural network in the vertebral brain, each individual neuron connects to various targets. It does so by extensive axonal branching and terminal axon ramification. Unlike the growth cone – the axonal guiding structure that only bypasses the target region, a branch reaches directly target zone that permits creating connexion via synapse (Lewis et al., 2013).

A branch is typically evoked from the collaterals of the axon shaft. This lateral protrusion occurs in a delayed time when a growth cone has already bypassed the target for a moment (Szebenyi et al., 1998). The delayed time can reach up to days. Notably, the growth cone typically pauses near the target zone: it repeatedly collapses and extends without any advancing forward. In dissociated neuron culture, growth cone pausing and delayed branch formation has also been observed. In cortical neuron culture, the growth cone undergoes a pause at the region where a branch develops after. During this pausing time, a modification occurs in the cytoskeletal structure of the growth cone zone, resulting in an extended region that is left behind when the growth cone moves forward. Subsequently, axon branches emerge from this active zone (Dent and Kalil, 2001).

Branch formation shares various similarities to axon guidance. Both begin with local extracellular cues in the target regions, which signals through axonal membrane receptors to activate intracellular signaling cascades regulating the cytoskeletal network. Four families of guidance cues have been identified, including netrins, semaphorins, slits, and ephrins. These guidance cues concurrently regulate the process of axon branching. Besides, axon branching is also modulated by other extracellular signals, including growth factors and morphogen (Dickson, 2002; Kalil and Dent, 2014).

3.2.2.2.2. Regulation of axon guidance and branching

Axon guidance cues

The guidance signals, often being glycoproteins secreted into the extracellular matrix, are ligands for transmembrane receptors on the surface of the axonal growth cone. In the presence of these ligands, their corresponding receptors are activated to trigger multiple intracellular responses, including membrane remodeling, cytoskeletal reorganization, and protein translation regulation. The collaboration between these intracellular signaling ultimately induces retraction or promotion of the growth cone.

Netrins

Netrin was the first identified neuronal guidance cue: its name is derived from the Sanskrit word *Netr*, meaning “guide”. Among the members of this family, netrin-1 is the most well-studied guidance cue. However, its roles are not only limited to axon guidance but also in axon branching, synaptogenesis, cell migration, etc. (Boyer and Gupton, 2018).

Netrin-1 is a diffusible chemoattractant that was first purified from chick brains (Serafini et al., 1994). It is secreted by floor plate cells and attracts the commissural axons in both the brain and spinal cord (Kennedy et al., 1994; Serafini et al., 1996). Netrin-1 also acts as a repulsive cue in some contexts. It has been described firstly in the trochlear neurons in vitro (Colamarino and Tessier-Lavigne, 1995). Among the two totally opposite, the ultimate effect is defined by which receptor is involved in the signaling process. Netrin interacts with two transmembrane receptors: Deleted in colorectal cancer (DCC) and Unc-5 (Mehlen and Mazelin, 2003). DCC is necessary for Netrin-1 axon promotion activity. Lack of DCC leads to loss of various commissures, including corpus callosum and hippocampal commissure (Serafini et al., 1996). In contrast, UNC-5 is required for the chemo-repellent activity of Netrin-1. Indeed, *unc5c* KO mice exhibit trochlear axon misprojections (Burgess et al., 2006). Furthermore, in vitro, the effect of netrin can be dependent on the extracellular environment. For instance, the presence of laminin leads to netrin-1-induced growth cone repulsion (Höpker et al., 1999).

In vitro, netrin-1 induces the formation of newly filopodial protrusion and prolongs the existent branches. When applied locally, netrin-1 induces the formation de novo of branches without impacting the main axon (Dent et al., 2004). Netrin-1 also increases terminal arborization by promoting arbor dynamic toward branch addition, resulting in an increase in total branch number (Manitt et al., 2009).

Semaphorins

Semaphorin (Sema) is a large protein family composed of both transmembrane and secreted proteins (Raper, 2000). It was originally identified in the invertebrate – in the CNS of grasshopper. Sema3A is the first discovered vertebrate member, which induces growth cone collapse in the dorsal root ganglion (DRG) culture (Luo et al., 1993). Semas are classified into eight classes: classes I-II are invertebrate proteins, classes II-VII include vertebrate proteins, and classes VIII are viral proteins. Among vertebrate Semas, only class III contains secreted proteins, while the remaining classes contain transmembrane proteins.

Most Sema proteins from class III, including Sema3A and D, mediate axon repulsion. Their activity has been demonstrated on various neuronal cells, including motoneurons, sensory, cortical and sympathetic neurons (Adams et al., 1997; Kobayashi et al., 1997; Varela-Echavarría et al., 1997; Bagnard et al., 1998). However, in some rare contexts, the repelling activity of Sema3 can be converted into an attractant, for instance, by activating the cGMP pathway (Song et al., 1998).

Sema3A is also involved in axonal branch sculpturing. Depending on neuronal cell type, effects induced by Sema3A are different. Sema3A inhibits axon branching in the cortical neuron culture and also decreases branch length without modifying the principal axon (Dent et al., 2004). In vivo, SEMA3A regulates the pruning of hippocampal axons (Bagri et al., 2003). However, with the GABAergic cerebellar cell, SEMA3A promotes branching. Mice with SEMA3A deficiency present a reduction in GABAergic axon terminal branching (Cioni et al., 2013).

Ephrins

Ephrin is a family of membrane-bound proteins which function as ligands of the Eph family of tyrosine kinase receptors. Ephrins and Eph receptors are arranged into two groups: ephrin As contains glycosylphosphatidylinositol membrane-anchored proteins, which bind to EphA receptors; and ephrin Bs contains transmembrane proteins and bind to EphB receptors (Lisabeth et al., 2013).

Ephrins are mostly linked to growth cone retraction in the developing neuron (Wahl et al., 2000; Murai et al., 2003). In vivo, Ephrins act as both attractant and repulsive cues. Ephrin A2 and A5 are essential for correct connection mapping of the anterior-posterior axis in the developing retina. The lack of these two proteins leads to the presence of additional arborization without

connected targets (Feldheim et al., 2000). In contrast, Ephrin Bs controls the retinotopic mapping in the dorsal-ventral axis and functions as an attractant of the interstitial branches (Hindges et al., 2002).

Ephrins can promote axon branching in a selective population of neurons. In the developing cortex, Ephrin A5 and its receptor are expressed in the distinct cortex layers. While it acts mainly as a repelling signal to other populations of neurons, it promotes the interstitial branch formation of layer VI neurons in the Ephrin A5-rich layers (Castellani et al., 1998). Through gradient distribution of Ephrins and their receptors, they promote the branch network only within the limit of the target zone.

Slit

Slit proteins are large secreted ligands of Roundabout (Robo) receptors. Slit and Robo were initially identified in *Drosophila* (Kidd et al., 1998, 1999). In *Drosophila*, the Slit-Robo complex induces axon repulsion from the midline (Battye et al., 1999; Kidd et al., 1999). This repelling action of Slit is conserved in vertebrates. In mammals, Slit proteins plays a role in the guidance of commissural neurons in the spinal cord and forebrain (Long et al., 2004; Sabatier et al., 2004; López-Bendito et al., 2007, 1).

Slits have been found to promote the axon branching in the DRG axon in vitro (Wang et al., 1999). In zebrafish, Slit proteins enhance the branching of trigeminal axons but inhibit this process in the retinal ganglion cell axons (Yeo et al., 2004; Campbell et al., 2007).

Growth factors

Growth factors, especially neurotrophins such as NGF and brain-derived neurotrophic factor (BDNF), promote both axon branching and arborization. BDNF evokes protrusions along the spinal axons of frogs (Gibney and Zheng, 2003). Moreover, BDNF also increases axon terminal arborization in retinal axons in the frog tectum (Marshak et al., 2007). A later study on mouse cortical neurons demonstrates that BDNF also induces axon branching in this population of neurons. Furthermore, this activity is mediated *via* the MAPK pathway (Jeanneteau et al., 2010). Different from netrin-1, BDNF promotes the formation of novel branches and stabilizes existing branches (Manitt et al., 2009).

Morphogens

WNT is a large family of secreted glycoproteins that play essential roles in embryonic development. Several WNT protein also regulates axon branching. WNT7A and WNT3A

function as axon branch inducers, while WNT5A increases the axon and branch length but not the number of branches (Hall et al., 2000; Krylova et al., 2002; Li et al., 2009).

3.2.2.2.3. Reorganization of the cytoskeleton

The cytoskeletal network is reorganized in response to extracellular signals, which induces branch formation. The initiation of branching begins with the appearance of filopodial and lamellipodial protrusions along the axon. Filopodia is a structure composed of unbranched actin filaments, while lamellipodial protrusions contain a meshwork of branched actin filaments. Accumulation of actin filament forming the protrusion is the initial step for branch formation (Figure 16) (Lewis et al., 2013).

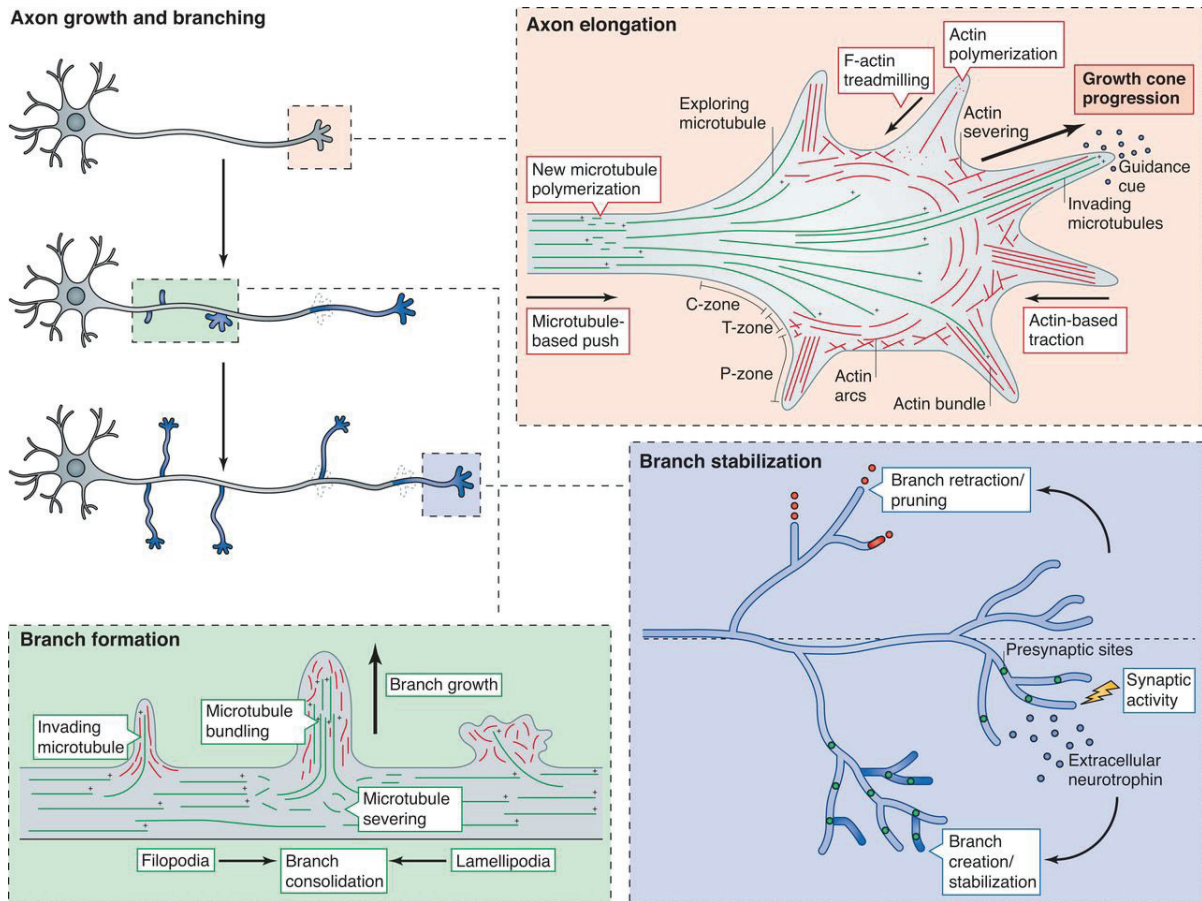


Figure 16. Cytoskeletal network reorganization during axon elongation and branching

Axon elongation is guided by the growth cone. Collateral branches often develop at the sites where the growth cone is paused (dotted growth cone). Red box is a magnification of the axon growth cone. The growth cone is formed by the microtubule and actin network, which is organized into three zones. The C-zone is composed of microtubules; the P-zone contains mainly actin bundles that form filopodium, and the T-zone that is between the C- and P-zone contains actin arcs. Upon axon elongation stimuli, microtubules invade into the filopodia, combining with the actin treadmilling to create a pushing force for growth cone extension. Green box is a magnification of axon branch formation. The branching process is initiated by actin-based filopodia or lamellipodia formation along the axon shaft. Subsequently, microtubules invade into the filopodia. Microtubule bundling induces the elongation of branches. In the later stage, branches can be stabilized and continue to arborize or retracted (blue box) (Lewis et al., 2013).

Nucleators

Formins nucleate straight, unbranched actin through recruiting complex actin – profilin. Also, formins permit the elongation of actin filament by staying on the barbed end to prevent CPs binding (Shekhar et al., 2015). They are essential to neurite initiation *via* regulating filopodia formation. In cortical neurons, ectopic expression of a formin mouse homolog of diaphanous 2 (mDia2) re-establish filopodia formation and neuritogenesis in the enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) deficient neurons (Barzik et al., 2014).

Actin-related protein 2/3 (Arp2/3), different from formins, has a crucial role in branched actin filament formation. It does so through binding to the side of the existing filament and initiating a new filament growth as a branch (Mullins et al., 1998). In sensory neurons, NGF treatment promotes localized protein synthesis of Arp2/3 subunits. This upregulation of the Arp2/3 complex is indispensable for filopodia formation and collateral branch initiation (Spillane et al., 2012). The role of Arp2/3 in growth cone motility is otherwise in debate (Strasser et al., 2004; Korobova and Svitkina, 2008).

A less unknown actin nucleator strongly expressed in the brain, named cordon-bleu, also plays a role in neuritogenesis and axonal branching. Cordon-bleu functions as a straight actin nucleator from the barbed of F-actin as formins. Down-regulation of this protein reduces neurite and axonal branch number. Otherwise, it does not significantly impact the major axon length (Ahuja et al., 2007).

Elongation factors

Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) is an actin elongation factor. This family is ubiquitous in mammals and localizes to actin-rich zones such as filopodial tips, lamellipodia, and cellular adhesions. Ena/VASP binds to profilin-actin and brings it to the barbed end of the existing filament. Even though Ena/VASP does not show nucleating activities, it stays at the barbed end and protects it from the capping proteins (Applewhite et al., 2007). VASP also protects actin filaments from severing by gelsolin (Barzik et al., 2005).

ENA/VASP proteins mediate Netrin-1-induced filopodia formation in the hippocampal neurons, regulating neurite formation, branching, and axon guidance (Lebrand et al., 2004).

Severing factors

Gelsolin is a potent actin severing factor. In the presence of calcium, gelsolin is activated and binds to the side of actin filaments, then severs the mother filaments into two shorter ones.

Gelsolin also covers the barbed ends of two daughter filaments and blocks G-actin monomer addition. In mice, gelsolin null neurons present more filopodia along the neurites (Lu et al., 1997).

Actin-depolymerizing factor (ADF)/cofilin is a family of small actin-binding proteins of 15-21 kDa. ADF/cofilin have a mild severing activity, independent of Ca^{2+} presence. Different from gelsolin, the barbed ends of two daughter filaments induced by ADF/cofilin are free without capping and freely develop. Aside from severing activity, ADF/cofilin rapidly depolymerizes actin filaments at the pointed ends. Hitherto, this is the only known protein family with significant actin depolymerization activity. Therefore, it has a critical role in regulating the cellular actin monomer pool. In retinal neuron culture, BDNF increases the number of growth cone filopodia and their length *via* activating ADF/cofilin (Gehler et al., 2004).

Microtubule dynamics

The emergence of a protrusion through filopodia is the first step of axonal branching. Nonetheless, not all filopodium afterward become mature branches. Actin network rearrangement creates the pushing force that forms the protrusions, but further development and stabilization require the formation of the microtubule network into filopodia (Brill et al., 2016). In the axon, microtubules form parallel bundles of filaments with the plus end towards the axon tips. Microtubule stabilization is a crucial step in defining an axon among multiple neurites (Witte et al., 2008).

Even though the microtubule network is stable along the axon, it is highly dynamic at the growth cone and novel protrusions along the axon shaft (Kahn and Baas, 2016). The remodeling of microtubules in the growth cone begins with the splaying off of the microtubule bundle into individual thin strands that allow them to invade the growth cone's protrusions. Engagement of microtubules in the growth cone is required for growth cone advance and axon elongation. Indeed, low doses of vinblastine induce axon growth cessation without impact on the pre-existing axonal microtubule structure (Challacombe et al., 1997). The individual microtubules then undergo a dynamic extension *via* polymerization. The microtubule network in the growth cone is present in various forms, which reflect the correlation between the growth rate of microtubules and the advanced move of the growth cone membrane. Microtubule looping occurs when the rate of microtubule extension exceeds growth cone advance (Dent et al., 1999). Aside from stabilizing newly axon compartments, the microtubule is thought to provide further

guidance to the growth cone from microtubule-carrying organelles, including mitochondria and ER (Dent and Gertler, 2003).

It happens similarly in the case of a branch formation: an actin composing protrusion is followed by microtubule invasion through splaying, looping, and bundling.

Microtubule development *via* polymerization, depolymerization, and severing is highly regulated by microtubule-binding proteins. In neurons, short microtubule fragments are synthesized by centrosome in the cell body and then transported to distal axon and neurites by motor proteins kinesins and dynein (Slaughter et al., 1997).

Microtubule plus-end tracking proteins (+TIPs)

Microtubules are composed of cylindrical hollow tubes that the wall is formed by dimer subunits of α - and β -tubulin. The tube is polarized, with a plus and a minus end. In living cells, polymerization occurs at the plus end, which is usually directed toward the cellular membrane (Desai and Mitchison, 1997).

+TIP is a diverse protein family composed of microtubule-binding proteins accumulated at the plus end. These proteins stabilize the plus end and often promote the growth of microtubule molecules (Kumar and Wittmann, 2012). They serve important roles in neuron polarization and axon guidance by the growth cone. Some are also involved in axon branching.

Adenomatous polyposis coli protein (APC) is enriched at the growth cone of the future axon among immature neurites, and its level is maintained high during neurogenesis (Koester et al., 2007; Shintani et al., 2009). Doublecortin (DCX) deletion results in more processes in primary neurites and a shorter leading process (future axon). DCX likely maintains an appropriate stable status for microtubule bundles, therefore preventing improper branch formation (Tint et al., 2009).

Severing proteins

Two microtubule severing proteins expressed in neurons are katanin and spastin. Katanin is widely distributed through the developing neuron, while spastin is accumulated at the branch site (Karabay et al., 2004; Yu et al., 2008). In addition, spastin expression is more specific in the neuron, while katanin is broadly found in many cell types (Baas et al., 2005; Ma et al., 2006).

Prior to branch formation, dramatic changes are observed in the microtubule network. Long strands of microtubule are locally disrupted into shorter fragments that are more mobile to invade the newly forming branches (Dent et al., 1999). Microtubule severing is therefore critical for branch formation.

Spastin downregulation decrease axon branches, and in contrast, spastin overexpression enhances collateral process formation. Solely katanin overexpression does not result in branch increase, but it needs to be combined with tau depletion (Yu et al., 2008). These observations suggest that two severing proteins function differently. Spastin directly cuts local microtubules at the branch point. Otherwise, katanin requires a local tau dissociation to promote branch formation.

Microtubule destabilizing proteins

Microtubule destabilizing proteins negatively regulate branch formation, which assures correct wiring of the neural network (Maor-Nof et al., 2013). Kinesin family member 2A (KIF2A) is a member of the kinesin superfamily. This protein is predominantly expressed in juvenile mouse brains. It hydrolyzes microtubule polymers using energy from ATP (Ogawa et al., 2004). KIF2A deletion in mice abolishes the suppression of the collateral branch's elongating part, which results in longer branches but no difference in branch number (Homma et al., 2003).

Microtubule-actin interactions

A protrusion formed by F-actin will not become a branch without microtubule presence. Nonetheless, a relatively long protrusion composed of actin and microtubule still possibly retracts and collapses. That means microtubule infiltration into a novel protrusion is necessary but insufficient for branch formation.

Microtubule and actin act together coordinately during the formation of the axon branch. Dynamic microtubule is co-localized with actin filament in the new protrusion. Drugs that inhibit either microtubule or actin polymerization also interrupt the remaining structure polymerization, resulting in branch formation diminution (Dent and Kalil, 2001).

In the growth cone, it exists a physical contact between actin filaments and growing microtubules *via* interaction between two binding proteins: end-binding protein 3 (EB3) and drebrin. Corruption of this interaction results in growth cone abnormalities or absence and impaired neuritogenesis (Geraldo et al., 2008).

3.2.2.2.4. *Signaling pathways*

Extracellular cues-induced modifications in the cytoskeletal network are mediated by signal transduction pathways. No single direct pathway is assigned to cytoskeletal regulation in developing neurons, including growth cone guidance and axon branching.

NGF promotes the formation of actin filopodia and branches in chick sensory axons *via* the PI3K pathway. NGF receptor tropomyosin receptor kinase A (TRKA) activates PI3K, which, in turn, activates Ras-related C3 botulinum toxin substrate 1 (Rac1) and then Wiskott-Aldrich syndrome protein-family verprolin homologous protein 1 (WAVE1). WAVE1 finally activates Arp2/3, an actin nucleator, to promote actin polymerization. Furthermore, BDNF and netrin-1 have been found to inhibit calpain-mediated cortactin degradation. Cortactin is an actin-associated protein stabilizing the actin filaments. Cortactin upregulation promotes actin polymerization and filopodia emergence (Spillane et al., 2012).

BDNF is also found to induce its activity on axon branching by regulating the MAPK pathway. BDNF induces upregulation of MAPK phosphatase-1 (MKP-1), which phosphorylatedly inactivates the MAPK and its downstream molecules, including JNK. JNK inhibition reduces the phosphorylation of stathmin-1 and activates this protein. Stathmin-1 is a microtubule destabilization that sequesters free tubulin. Overall, BDNF induces microtubule destabilization, which is likely required for BDNF-induced axon branching (Jeanneteau et al., 2010).

In addition, WNT proteins may also mediate their activity through GSK3 β signaling. When WNT inhibits GSK3 β , the phosphorylation of MAPs, including tau, MAP A, MAP B, and APC, is dampened. A decrease in MAP1B phosphorylation would increase microtubule stability, which is important to promote the growth of the nascent branch (Salinas, 2007).

3.2.2.2.5. *Calcium signaling in the axon guidance and branching*

Ca²⁺ signaling plays an essential role in transducing the extracellular cues into cytoskeletal reorganization (Kalil and Dent, 2014). Both actin filaments and microtubules are sensitive to intracellular Ca²⁺ concentration change. However, the mechanism by which Ca²⁺ regulates the cytoskeletal network in general, and in particular during axon development, has not been fully clarified.

Notwithstanding, there is emerging evidence that the amplitude of cytosolic Ca²⁺ spike is important to interpret an extracellular cue as an attractant or repulsive. A high peak of Ca²⁺ will activate CaMKII, which subsequently supports the axon or its branches to move forward. Conversely, a relatively low Ca²⁺ increase activates instead CaN and protein phosphatase-1

(PP-1), which leads to axon retraction (Wen et al., 2004). Such protein sensing Ca^{2+} change system also regulates the dendritic spines (Fujii et al., 2013).

Ca^{2+} -regulating the cytoskeletal network is mainly *via* phosphorylation regulation. An essential target of CaMKII is the family of Rho GTPase. Due to the phosphorylation activity of CaMKII, different Rho GTPase can be activated, including RhoA, Rac, and cell division cycle 42 (Cdc42) (Jin et al., 2005). Rho GTPases promote actin polymerization through activating two groups of actin-binding proteins. They activate WAVE proteins, which, in turn, activate the Arp2/3 complex (Pollard and Borisy, 2003). Rho GTPase proteins also activate Diaphanous-related formins (DRFs), a well-known family of formins (Kühn and Geyer, 2014). Overall, Rho GTPases promote both actin polymerization on existent F-actin and new branch formation. Concurrently, Rac1 and cell division control protein 42 (Cdc42) inhibit the activity of cofilin by activating its inhibitory kinase LIM kinases (LIMKs) (Edwards et al., 1999). In contrast, CaN has been found to activate slingshot, a phosphatase that can activate cofilin. That would partly explain the repelling effect induced by CaN (Wang et al., 2005).

3.2.2.3. Synaptogenesis

3.2.2.3.1. Synaptogenesis – a complex multi-step process

The synapse formation occurs in the early development, extending from the embryonic stage into the early post-natal period. Evidence shows synaptogenesis maintains throughout the lifetime of an organism, relating closely to learning and memory. A synapse is frequently a functional communicative contact from an axon of a neuron toward a dendrite of another neuron. However, a synapse can also be established between an axon with the soma or the axon hillock.

During the initial stage of synaptogenesis, an axon must extend to reach its target that can be located far away. When the presynaptic and postsynaptic membranes are determined and approached, physical connections are established between two neurons *via* trans-synaptic cell adhesion molecules (CAMs), neuroligin-neurexin, and cadherins. Subsequently, essential structures are accumulated and assembled at both presynaptic and postsynaptic membranes (Okabe et al., 2001). The components required for neurotransmitter synaptic vesicle (SV) transmission are recruited and centralized into the active zone (AV) at the presynaptic. The AV is a disc-like region located in the central presynaptic part of the synapse. It is composed of a highly dense protein matrix that contains five core proteins: Rab3-interacting molecule (RIM), mammalian uncoordinated 13 (Munc13), α -liprin, RIM binding protein (RIM-BP), and ELKS.

These proteins form a large scaffold complex that anchors the SVs and the Ca²⁺ channels, which control the SV exocytosis, and docks these structures to trans-synaptic adhesion molecules (Südhof, 2012; Rizalar et al., 2021). At the postsynaptic part of a synapse, neural transmitter receptors are recruited to scaffold postsynaptic protein into a specialized region called postsynaptic density.

The initial assembly of the synapse is followed by its maturation process to acquire its mature structure and functional properties. The maturation process of glutamatergic synapses – the most common excitatory synapses in the brain, involves the formation of small protrusions called dendritic spines that harbor the postsynaptic density. This formation of dendritic spines, also called spinogenesis, is an important event featuring synapse maturation. Concurrently, protein recruitment is continued, and the amount of both pre-and postsynaptic components increases substantially. Some synaptic components are recruited only to synapse during the maturation, such as AMPAR (Washbourne et al., 2002).

3.2.2.3.2. Spinogenesis – synapse maturation

Dendritic spines are small protrusions on the dendrites. This structure houses the majority of excitatory glutamatergic synapses (Calabrese et al., 2006). During early neuronal development, immature glutamatergic synapses are principally located at the dendritic shaft (Reilly et al., 2011). Concomitantly with the maturation process, spine synapses increase, and shaft synapses drop. In contrast, most inhibitory synapses are located at the dendritic shaft and the cell body.

Spine shapes are classified into four groups: stubby, thin, mushroom, and filopodium (Peters and Kaiserman-Abramof, 1970). Mushroom-shaped spines represent the most common type of dendritic spines in mature pyramidal neurons. It is not clear what are the roles of different forms of spines. Two main hypotheses possibly explain this phenomenon. Firstly, this perhaps reflects the multi-step formation of the spine during which the immature spines undergo morphological changes into mature mushroom-shaped spines (Yuste and Bonhoeffer, 2004). Secondly, the presence of various spine shapes may indicate morphology-related synapse plasticity. The two models do not rule out each other and are possibly both the origins of spine morphological diversity (Borczyk et al., 2019).

Three models of spine formation

Spinogenesis begins at the early stage after birth in many species. In rats, spine formation initiates at the post-natal week 1. During four following weeks, spine density increases gradually and then reduces with increasing age (Yuste and Bonhoeffer, 2004).

Besides the change in density, dendritic spines undergo morphological modifications during development. Stubby spines are commonly found in the early stage of development (Miller and Peters, 1981). In adults, thin and mushroom-shaped spines are more common.

According to the model proposed by Miller/Peters, spinogenesis is a three-step process (Miller and Peters, 1981). First, a synapse is formed at the contact between the axon tip with the dendritic shaft. From this synapse, an immature spine emerges under the stubby form, without a clear neck. This stubby spine subsequently evolves into thin or mushroom-shaped spines. This model highlights the role of the axon terminal on spine induction.

The model proposed by Sotelo developed from the observations in Purkinje cells undermines the role of the axon terminal on spinogenesis. This hypothesis suggests that the spine emerging depends not on the axonal terminal. When an immature spine protrusion encounters an axon, a synapse develops on this spine that induces spine shape change subsequently (Sotelo, 1990).

These two models do not explain the existence of filopodial spines. Dendritic filopodia are elongated (2-20 μm), thin ($>0.3\ \mu\text{m}$ in diameter) protrusions. In normal conditions, filopodia are rare on mature neurons, which proposes their roles are mainly developmental. Based on Vaughn's model developed in Purkinje cells, dendritic filopodia capture the surrounding axons. The filopodium successfully grabbing an axon subsequently develops into a synapse-bearing spine (Vaughn, 1989).

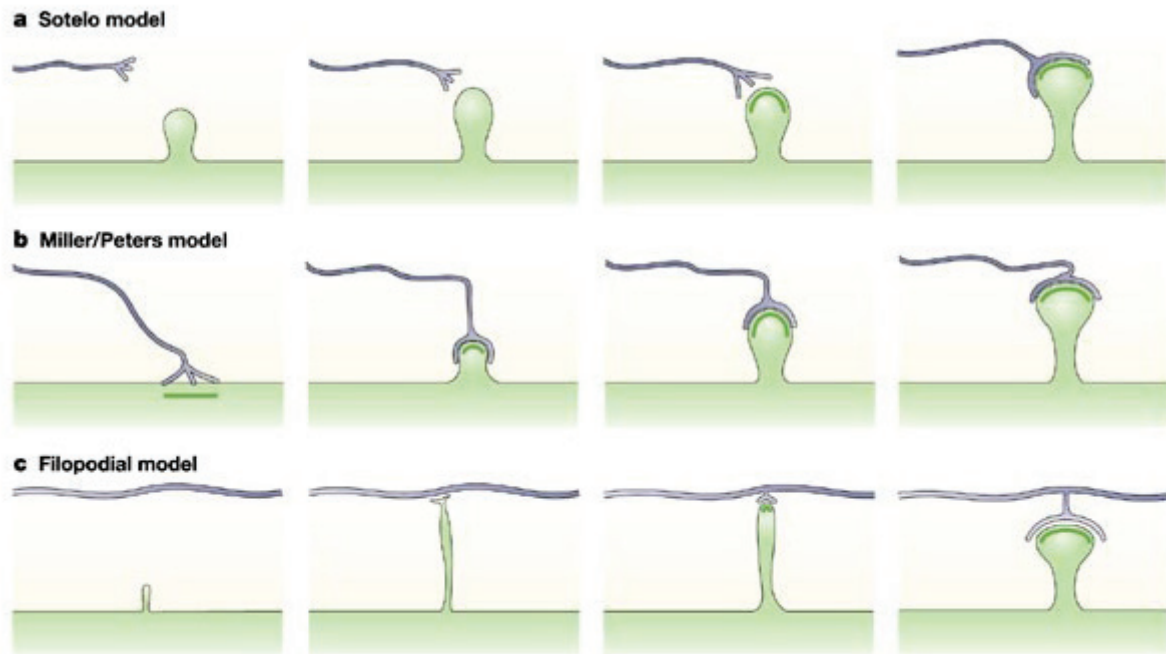


Figure 17. Three models for spinogenesis

(a) In the Sotelo model, dendritic spines emerge independently of the axonal terminals. When an immature spine is contacted by an axon terminal, it develops into a mushroom-like mature spine. **(b)** In Miller/Peters model, dendritic spines only develop in the presence of axon contact. **(c)** In the filopodial model, spines are pre-existent under the filopodial form. This spine pre-form can elongate to search for axon contact. When a spine reaches an axon, it develops into a mushroom-like spine (Yuste and Bonhoeffer, 2004).

3.3. Apoptosis in the development

Cell death is essential for animal development, including establishing and maintaining tissues and organs. It would not exaggerate to state that the key principle of metazoan development is the over-production of cells *via* proliferation at the early stage, followed by the trimming process *via* apoptosis or other programmed cell death at the later stage (Jacobson et al., 1997). During development, various structures are formed initially and are later removed by apoptosis. These structures, usually functional in ancestral organisms but becoming now unuseful for modern species, are eradicated by apoptosis during development. For instance, the interdigital membranes present in the amphibian are eliminated during the embryonic stages of amniotes (Hernández-Martínez and Covarrubias, 2011). That also causes flexibility to adapt basic structures to different sexes and stages of development (Meier et al., 2000).

In mammals, apoptosis displays vital roles, especially in the development of the nervous system and immune system, which are two systems presenting a high order of complexity (Meier et al., 2000). For instance, during the lymphocyte T development, they undergo two scrupulous selection processes. The first occurs just after the rearrangement of the antigen receptor genes. Only about 1/3 with successful antigen receptor formation survive through this first wave of elimination. Consecutively, T-lymphocyte progenitors with a functional antigen receptor are under the second selection to eliminate cells bearing self-reactive antigen receptors. These extremely selective processes create a tremendous diversity of responding capacity of the immune system and concurrently protect the body from potential attacks by its own immune system (Opferman, 2008).

Apoptosis in the developing nervous system

Apoptosis is known to play essential roles in the development of the CNS. Indeed, cell death events have been largely described in the brain of vertebrate embryos, whereas experimental suppression of apoptosis in the developing embryo mainly results in deleterious malformations in the nervous system (Yamaguchi and Miura, 2015).

Control of neural tube closure

Numerous apoptotic events occur during the nervous system development in vertebrates and invertebrates. At an early developmental stage, apoptotic cells are found at the boundaries between the non-neural ectoderm and the neuroepithelial layer during and after neural tube closure (NTC) (Geelen and Langman, 1977). Interestingly, apoptosis takes place throughout the major steps of NTC, including neural fold bending and fusion processes, remodeling of the

dorsal neural tube and the ectoderm, as well as migration of neural crest cells away from the neural tube (Massa et al., 2009; Washausen et al., 2018). Indeed, suppression of apoptosis with caspase inhibitors entirely impedes the NTC in the chick embryo (Weil et al., 1997). Furthermore, there is evidence of a potential link between mutations in apoptosis-related genes (*caspase-3*, *caspase-9*, *apaf1*) and neural tube closure defects in humans (Liu et al., 2018; Spellacy et al., 2018; Zhou et al., 2018).

In mice, *caspase-3*- and *apaf1*-null embryos only show neural tube closure defects in the midbrain and the hindbrain, while neurulation proceeds normally in the forebrain and the spinal cord. Interestingly, pharmacological inhibition of apoptosis using the pan-caspase inhibitor z-VAD-FMK or the p53 inhibitor Pifithrin- α does not impact neural tube closure in ex vivo cultured embryos (Massa et al., 2009). This observation suggests that the observed neural tube closure defect may not be a direct consequence of the suppression of apoptosis but could be due to the abnormal persistence of certain signal-secreting cell populations that apoptosis should have eliminated. Indeed, this may explain why neural tube malformations were not observed in ex vivo cultures, where secreted factors are expected to be rapidly diluted in the culture medium.

Control of cell number

Apoptosis is widely recognized as the main regulator of cell number during development. In developing CNS, neurons are generated excessively. Then, this population is trimmed down *via* apoptosis: neurons without proper connection with their target are eliminated. In the context where apoptosis is suppressed or compromised, the nervous system, especially the brain, would be overloaded with neurons, leading to protrusions and ventricular obstruction (Jacobson et al., 1997; Buss et al., 2006).

Several KO mouse models demonstrated the critical role of caspase-dependent apoptosis in controlling the size of neuron populations during development. Caspase-3 invalidation in 129X1/SvJ mice leads to exencephaly, a defect in which the brain exhibits abnormal protrusions and ventricular absence due to hyperplasia (Kuida et al., 1996). Moreover, caspase-9-null embryos showed prenatal malformations in the brain and the spinal cord, similar to those observed in caspase-3-null embryos, including protrusion formation and ventricular obstructions. Both caspase-3- and caspase-9-null mice die rapidly after birth (Kuida et al., 1998). Of note, these mice show an abnormal increase in neuron number, suggesting that MOMP is indeed required for neuronal death during the development of the CNS. Interestingly, invalidation of *apaf1*, a gene coding for a key component of the apoptosome, results in a more

severe hyperplasia phenotype than in caspase-3- or caspase-9-null mice, further supporting the role of the intrinsic apoptosis pathway in brain homeostasis (Cecconi et al., 1998; Yoshida et al., 1998). Finally, mouse embryonic fibroblasts derived from cytochrome c-null embryos failed to activate caspase-3 in response to different apoptotic stimuli. Of note, the later embryos exhibited delayed development, dying at E10.5 (Li et al., 2000). However, it remains unclear if these effects are due to apoptosis failure or mitochondrial electron transport chain deficiency.

Regarding the extrinsic pathway of apoptosis, even though caspase-8 invalidation causes heart malformation, erythrocyte congestion, and neural tube defects, leading to embryonic death at E13.5, the malformations observed in the nervous system are conceivably not directly linked to caspase-8 silencing (Varfolomeev et al., 1998; Sakamaki et al., 2002).

It should also be noted that malformations of the neural tube induced by the invalidation of *caspase-3* and *apaf1* are mouse-strain specific. Indeed, contrary to 129X1/SvJ mice, *caspase-3*-null C57BL/6J mice showed no macroscopic anomalies and reached adulthood without any detectable brain pathology (Leonard et al., 2002). A similar strain-dependent effect was also observed in *apaf1*-null mice. In effect, C57BL/6J and CD1 mice show significantly delayed lethality compared to 129/Sv mice. These controversial observations might be due to some redundancy between effector caspases. Actually, although neither caspase-6 nor caspase-7 appears to be critical for CNS development, caspase-7 is capable of triggering apoptosis in a caspase-3-independent manner and is potentially responsible for the “rescued” phenotype in C57BL/6J caspase-3-null mice (Houde et al., 2004; Lakhani, 2006; Uribe et al., 2012).

Finally, although this issue was not addressed in the above studies, compensation by other types of PCD such as autophagy and necroptosis cannot be eliminated.

The notion that, during development, cell number is exclusively controlled by apoptosis has recently been challenged by Nonomura and colleagues (Nonomura et al., 2013). In this study, apoptosis suppression by *caspase-9* or *apaf1* invalidation, even in a 129S1 background, did not increase nerve cell number, whereas severe brain malformations were still observed. Actually, the size of neuron populations would be controlled by proliferation rate and other types of PCD.

In contrast, apoptosis was shown to be essential for controlling the fibroblast growth factor 8 (FGF-8)-producing cell population. Indeed, after the NTC, apoptosis is maintained in the midline of the neural tube, where these cells are localized. During development, activation of apoptosis around day E10 leads to the removal of these FGF-8-producing cells, preventing ectopic diffusion of FGF-8 to the ventral telencephalon (Nonomura et al., 2013). Together,

these observations support the notion that the roles of apoptosis in the developing nervous system are actually cell-type specific.

3.4. Mitochondria in neural development

The mitochondrion is the primary energy factory of eukaryotic cells. It generates ATP in the most effective way *via* OXPHOS.

At rest, the brain demands approximately 20% of the body's ATP, which is up to 50% in children (Kuzawa et al., 2014). This significant demand is due to the complex morphology of neurons, multiple highly maintained ion gradients, and thousands of synapses in constant activities in each neuron. Neurogenesis initiation is marked by a metabolic program transition of neural progenitor cells from glycolysis into mitochondrial-dependent OXPHOS (Zheng et al., 2016). These metabolic changes are illustrated by the mitochondrial morphological shift towards a more elongated shape and the upregulation of OXPHOS-related genes such as the complex V encoding gene.

During neurogenesis, post-mitotic immature neurons undergo cellular polarization, with a long axon and multiple dendrites formation and a highly complex branch network afterward. Process elongation and branching, and synaptogenesis processes require a great amount of ATP to create and reorganize cytoskeletal structures. Accordingly, the neuron is under challenge to satisfy energy requirements in the cellular compartments that are usually far from the cell body. Mitochondria are mobilized and widely distributed into axon and dendrite networks to reply rapidly to these local needs (Sheng, 2017). Thus proper localization of mitochondria is critical for neural development. The mechanisms of mitochondria distribution in neurons are far from well-understood. Recent work unveils some elements of this highly regulated process.

Mitochondria are transferred to distal processes along microtubules *via* motor proteins of the kinesin or dynein family. In the axon, kinesin motors move towards the distal end, and dynein motors move backward to the cell body. Miro, a MOM protein, connects with motor protein through the adaptor, Milton/TRAKs proteins. Miro 1 and 2 are Ca^{2+} - binding GTPase proteins, regulating mitochondria transporting. When calcium concentration is elevated, Miro changes its conformation, which disrupts the interaction between Miro with motor proteins or motor proteins with microtubules (MacAskill et al., 2009; Wang and Schwarz, 2009). Calcium-related morphological dependency of Miro provides a potential mechanistic regulation over mitochondria motility. The current model proposes that the activity of synapses that induce a Ca^{2+} rise produces a signal to halt surrounding mitochondria (MacAskill et al., 2009). However, whether this mechanism determines mitochondria distribution at the branch points is still an open question. In addition, recent evidence shows that mitochondria motility drops drastically,

and they lose their sensitivity to Ca^{2+} in the mature neurons (Lewis et al., 2016). That means other mechanisms must also involve in shaping the mitochondria network.

In the developing neuron, only approximately 30% of axonal mitochondria are motile at a given time, while others stay stationary. The docking status of axonal mitochondria is maintained by an axon-specific protein named syntaphilin. This protein is present in about 65% of axonal mitochondria, corresponding with the immobile mitochondria population. Its loss results in motility increase and density diminution of mitochondria (Kang et al., 2008). Besides syntaphilin, the serine/threonine kinase Liver kinase B1 (LKB1) and its downstream Nuclear AMPK-related kinase 1 (NUAK1) are also implicated in the mitochondria docking. Loss of LKB1 or NUAK1 induces a decrease in stationary mitochondria to a similar level observed in Syntaphilin-null neurons (Courchet et al., 2013). However, hitherto there is no evidence that syntaphilin is regulated by the LKB1-NUAK1 pathway.

Mitochondria roles in the neuron

Mitochondria play critical roles in the neuron development and the normal function of mature neurons. Mitochondria axon positioning is dispensable for axon branching and synapse formation (Li et al., 2008a; Lewis et al., 2018). Loss of mitochondria at synapses leads to a less efficient response to stimuli (Lewis et al., 2018).

ATP production

OXPPOS is executed by four complexes (I-IV) located in the MIM, which continually transfer electrons through the ETC and concurrently pump protons from the mitochondrial matrix into the intermembrane space. The activities of the ETC result in an accumulation of protons in the IMS. The re-entry of protons back to the matrix *via* ATPase synthase (or complex V) leads to ATP production (Formosa and Ryan, 2018).

During axon branching, mitochondria are driven from the cell body to axon terminals and branching points in response to energy needs. The mitochondrial uncoupling FCCP that inhibits mitochondrial ATP production dampens the axon branching, which confirms a causality between local mitochondria-generating ATP and axon arborization (Tao et al., 2014). In the axon, mitochondria are docked at the “hot spot” of axonal protein translation. Mitochondria respiration collapse impairs protein synthesis in the axon and axon branching (Spillane et al., 2013).

Calcium buffering

Mitochondria also play roles in intracellular calcium buffering. In response to Ca^{2+} increase, mitochondria may accumulate 10 to 20 times more Ca^{2+} inside their matrix than cytosol (Duchen, 2000). Mitochondrial Ca^{2+} uptake is rapid and active. To enter the mitochondrial matrix, Ca^{2+} is actively transported *via* VDACs at the MOM and the mitochondrial Ca^{2+} uniporter (MCU) complex at the MIM (Patron et al., 2013; Rosencrans et al., 2021). Mitochondria also receive directly Ca^{2+} flux from ER *via* MERC.

Role Ca^{2+} homeostasis is highlighted in the function of the synapse, especially for the SV release. Reduced mitochondrial calcium uptake perturbs the SV release and short-term plasticity (Kwon et al., 2016).

Whether Ca^{2+} -related functions of mitochondria are involved in neuron development remains obscure. Notwithstanding, as Ca^{2+} plays an important role in the cytoskeletal network regulation, this possibility is plausible.

Caspase activation

Mitochondria are also capable of triggering the caspase activation cascade and ultimately leading to caspase-3 activation. It is noteworthy that emerging evidence proposes that caspase-3 activation is not obligatory to be a sign of inevitable cell death as described in the traditional model. A sub-lethal caspase-3 activation may be a result of the incomplete MOMP phenomenon, as discussed previously.

Emerging evidence suggest that caspases play essential roles in the development of neuron, which we will discuss in detail in the next part. Interestingly, most studies showed the critical role of the intrinsic pathway of caspase activation, therefore mitochondria role, in generating this physiological caspase signal (Nikolaev et al., 2009; Guo et al., 2016).

3.5. Non-apoptotic roles of caspases in the developing nervous system

3.5.1. Axon branching and arborization

Recent studies proposed that caspases, particularly caspase-3, play multiple roles aside from apoptosis. Activation of caspase-3 has been observed transiently and locally in the axons, notably at the level of branching points (Campbell and Okamoto, 2013; Katow et al., 2017). Intriguingly, this transient activation of caspase-3 did not always lead to apoptosis of the host neuron. Inhibition of caspase-3 in these models resulted in a decrease in the number of axonal branches. Caspase-3 cleavage also mediated the growth cone formation (Campbell and Holt, 2003; Verma, 2005)(Campbell and Holt, 2003; Verma, 2005).

The role of caspase-3 in axon regeneration was further highlighted in a model of axon disruption in *Caenorhabditis elegans*. In the latter, the homolog of vertebrate caspase-3 is encoded by the *ced-3* gene. Using neurons from a *ced-3*-null *C. elegans* strain, Pinan and colleagues showed that axons had a lower outgrowth rate and were unable to re-connect after axotomy (Pinan-Lucarre et al., 2012). In *C. elegans*, Wang and colleagues recently detected increased *ced-3* activity following axotomy, which was correlated to axon regeneration (Wang et al., 2019). Moreover, in neurons derived from rat DRG, caspase-3 inhibitors impacted growth cone formation, which is critical for restoring injured axons (Verma, 2005).

The mechanisms by which caspase-3 regulates growth cone formation and axon branching are still elusive. However, the contribution of the cytoskeleton is presumably important. In axons and dendrites, cytoskeleton components comprise actin microfilaments, microtubules, and intermediate filaments. In developing neurons, actin filaments are mainly found in growth cones. In contrast, microtubules are evenly distributed along the axons. However, it should be noted that the rate of microtubule remodeling is much higher in the axon terminus compared to axon shafts. The growth cone is a guiding structure at the terminus of developing axons, characterized by actin-enriched structures referred to as filopodia and lamellipodia (Pacheco and Gallo, 2016). Most interestingly, cytoskeletal actin was reported to be a substrate for caspase-3. Actin cleavage by caspase-3 gives rise to a 15kD fragment inducing a more condensed and fragmented actin network (Mashima et al., 1999).

Moreover, caspase-3 was demonstrated to cleave Rho-associated kinase I (ROCK I), generating a truncated form with higher intrinsic kinase activity. Such activated ROCK I stabilizes actin microfilaments, phosphorylates myosin light chains, and promotes the coupling between actin and myosin filaments, causing cellular contractions (Coleman et al., 2001). Thus, in the context

of neuronal cells, caspase-3 may exert the same function in order to stabilize the cytoskeleton of new axon branches.

Aside from actin microfilaments, intermediate filaments are also potential targets of caspase-3 in axons. Immature neurons mainly express vimentin and nestin as intermediate filaments that are replaced by neurofilaments in mature neurons. Aside from their role as scaffolding structures, vimentin, and nestin function as adaptors, contributing to axon growth cues (Adolf et al., 2016; Bott et al., 2019).

In addition to the cytoskeleton, caspases may further contribute to axon guidance through their impact on cell adhesion molecules, such as neural cell adhesion molecule (NCAM) and extracellular vesicle proteins (Westphal et al., 2010; Weghorst et al., 2020).

3.5.2. Axon pruning

Axon pruning is a process that eliminates collateral extensions or small terminus arborization with improper connectivity at the axon terminus. This process occurs at the embryonic or early postnatal stage to fine-tune neural connectivity and has been well described in the PNS. Axon pruning can be reproduced in vitro by withdrawing trophic factors such as NGF or BDNF from neuron cultures, including DRG. In the developing CNS, although still poorly understood, similar mechanisms of network fine-tuning may occur at the early postnatal stage (Innocenti and Price, 2005; De León Reyes et al., 2019).

In the sympathetic and DRG models, caspase-6 is widely recognized as an active contributor to axon pruning (Nikolaev et al., 2009; Cusack et al., 2013; Unsain et al., 2013). In contrast, the role of caspase-3 was initially overlooked. In the study of Nikolaev and colleagues (Nikolaev et al., 2009), activation of caspase-3 was not detected in axons using an immunofluorescent marker or the fluorescent reporter FAM-DEVD-fmk in sympathetic neurons in culture following NGF withdrawal. Furthermore, the inactivation of caspase-3 with pharmacological inhibitors or siRNAs did not prevent caspase-6 cleavage and axon degeneration.

However, recent reports from different laboratories highlighted the implication of caspase-3 in axon pruning using genetic approaches. These latter studies demonstrated that caspase-3 KO prevented axon degeneration in NGF-deprived neurons. Interestingly, in vitro studies showed that caspase-6 can only be processed by caspase-3, even at low levels of active caspase-3 (Simon et al., 2012; Cusack et al., 2013; Unsain et al., 2013). The difference between these observations and the ones of Nikolaev might be due to the small amounts of activated caspase-3 in this context, which cannot be detected by immunofluorescence. In fact, small remaining

amounts of active caspase-3, having escaped pharmacological inhibitors or siRNA, are presumably sufficient to promote the observed caspase-6-dependent pruning.

The roles of caspases in axonal network fine-tuning remain to be further investigated. Recently, regarding *in vivo* studies, caspase-3 is activated in the axon and not in the cell body of spinal cord neurons of postnatal mice. Suppression of caspase-3 activation by *bax/bak* DKO led to a less tailored network of neuron branches in the spinal cord at postnatal day 14, which disturbed the reorganization of the corticospinal circuit and consequently impaired the development of skilled movements in adult mice (Gu et al., 2017).

3.5.3. Synapse maturation and synaptic functions

Recent studies have suggested that, aside from refining the developing axonal network, caspases also promote fine-tuning of protein structure at the level of the synapses, which may be critical for their functioning. Synapses are the key player structures regarding neuron communication. There are two existing types of synapses referred to as electrical and chemical, the latter being the most abundant in the CNS. In electrical synapses, signals are transmitted by an electrical current through gap junctions. In chemical synapses, communication between two neurons is performed by the secretion of a neurotransmitter in the synaptic cleft. Once released into the synaptic cleft, the neurotransmitter binds to receptors at the post-synaptic level and induces electrical signals by acting on ion channel permeability (Purves et al., 2018).

In the PNS, caspase-3 fine-tunes the post-synaptic neurotransmitter receptor network. During development, this process namely occurs at the neuromuscular junction regarding the nicotinic acetylcholine receptor (AChR). Indeed, at the early stages of development, AChRs are abundantly distributed. Later on, AChRs gather into clusters that are stabilized by agrin released from axon terminals. Moreover, motor neurons secrete acetylcholine, which acts as a negative signal, disrupting AChR clusters that do not interact with axon terminals. Of note, this disrupting effect of acetylcholine is mediated by caspase-3, which cleaves Dishevelled segment polarity protein 1 (Dvl1), a signaling protein of the Wnt pathway. Indeed, prevention of caspase-3 activation or Dvl1 cleavage suppresses acetylcholine-dependent AChR cluster disruption (Wang et al., 2014).

Certain similarities between synaptogenesis processes in the CNS and the PNS have been described, such as the clustering of neurotransmitter receptors at the post-synaptic level (Garner et al., 2002; Purves et al., 2018). Regarding the underlying molecular mechanisms, agrin-like molecules and Ephrin-Bs have been reported to contribute to the clustering of NMDA and

AMPA receptors, respectively (Garner et al., 2002). It remains to be explored if the role of caspase-3 in sculpturing post-synaptic receptor clusters in the PNS is conserved in the CNS.

Caspase-3 is also involved in synaptic plasticity, notably in LTD, a process during which the efficiency of synaptic transmission is lowered in the long term. This process was thoroughly studied in the context of the glutamatergic synapse. The post-synaptic region of these synapses contains AMPA and NMDA receptors that both respond to glutamate. Coordination between both receptors is critical for optimal synaptic transmission. In LTD, the AMPA receptor is withdrawn from the post-synaptic membrane by endocytosis, which leads to a decrease in synaptic sensitivity (Purves et al., 2018).

Interestingly, caspase-3 activation is essential for AMPA receptor internalization at the synapse in response to NMDA stimulation *in vitro* (Li et al., 2010). Inhibition of the XIAP caspase-3 inhibitor enhances AMPA receptor internalization and increases LTD (Gibon et al., 2016). In addition, caspase-3 mediates synapse loss upon long-term exposure to NMDA (Henson et al., 2017). Furthermore, local activation of caspase-3 induced by Mito-killer Red photo-stimulation results in local spine shrinkage and subsequent elimination of spines without neuronal apoptosis (Erturk et al., 2014). Dendritic spine regulation by BDNF is also mediated by caspase-3 (Guo et al., 2016). *In vivo*, *caspase-3* KO mice display a lack of attention and hyperactive disorder, potentially related to a failure in synaptic plasticity mechanisms, such as AMPA receptor regulation in response to chronic or repeated stimuli (Lo et al., 2015). In contrast, *xiap* KO mice exhibit better learning performance, which could be explained by increased LTD (Gibon et al., 2016).

In addition, caspase-3 regulates the synaptic vesicle pool and eliminates dysfunctional dendritic spines (Chen et al., 2020). Collectively, these data highlight the central roles of caspase-3 in synaptic plasticity and functions.

3.6. Bcl-2 proteins in the development

3.6.1. Bcl-2 expression during the development

Bcl-2 proteins are highly expressed during mammalian development in general and, in particular, mouse development (Opferman and Kothari, 2018). Expression of anti-apoptotic and pro-apoptotic is kept in balance for normal development: a minor change can cause catastrophic abnormalities and ultimately results in death. Indeed, double loss of single alleles of *mcl1* and *bclx* induced craniofacial anomalies and neonatal death. The normal development can be rescued by a simple additional loss of a single allele of *bim* (Grabow et al., 2018).

Bcl-2 protein expression is detected from the early stage of development since the embryonic genome becomes active. Mcl-1 and Bcl-xL, Bax is highly expressed during development, while Bcl-2 expression is low during early embryogenesis and increased in the later stages (Metcalf et al., 2004). Even though their expression is observed from the early blastocyst, only Mcl-1 plays a critical role at this stage. Among genetically KO mice of Bcl-2 proteins, only *mcl1* KO dies at the peri-implantation stage (E3.5). However, without evidence of increased apoptosis, this lethal phenotype is likely due to non-apoptosis related role of Mcl-1 (Rinkenberger et al., 2000).

As discussed in the previous part, apoptosis has a pivotal role in the development of the nervous system and the hematopoietic lineages. Accordingly, genetic deletion of Bcl-2 proteins mainly results in abnormalities in the nervous system or the hematopoietic system.

In the developing CNS, Bid, Bax, Bak, Bcl-xL, and Mcl-1 are expressed at a high level (Lindsten et al., 2000; Krajewska et al., 2002; Arbour et al., 2008; Nakamura et al., 2016). Bcl-2 is also found to be highly expressed during neural tube formation (Michaelidis et al., 1996; Krajewska et al., 2002). Bcl-xL is the only anti-apoptotic protein whose expression remains elevated even in the postnatal brain.

3.6.2. Bcl-2 proteins in the development: focus on the nervous system development

As mentioned at the beginning, apoptosis may take place in healthy cells, in which it is programmed as a “predestination” to ensure the morphogenesis and normal development, but also to preserve the tissue homeostasis and function (Suzanne and Steller, 2013; Shalini et al., 2015). Bcl-2 proteins, as apoptosis regulators, are thought to be strongly implicated in the development. Thanks to genetically modified mouse models, the implication of almost Bcl-2

proteins is decoded. Here, we review some of the most important aspects, with a focus on the development of the nervous system (Table 2).

3.6.2.1. Pro-apoptotic proteins

Single invalidation of pro-apoptotic proteins does not dampen the normal development. That suggests that the roles of apoptosis accelerators in the development are redundant.

Bax invalidation does not entirely eliminate apoptosis during development. Homozygous *bax* KO mice are *viable* but exhibit anomalies in some specific cell populations. The lymphocyte T and B population is expanded without control. Increased granulosa cell number forms abnormal follicle structure in the ovaries (Knudson et al., 1995). The presence of *Bax* is also critical for the development of some specific neural populations, including neuroblasts, retinal ganglion cells, and Cajal-Retzius neurons (Péquignot et al., 2003; Ledonne et al., 2016). *Bak* KO mice develop normally without detectable malformation in any tissues, including the CNS (Lindsten et al., 2000).

DKO of *bax* and *bak* leads to a considerably more severe phenotype. DKO mice are born with interdigital membranes and an imperforate vagina. Most homozygous DKO mice fail to nurse correctly and die within 48h of birth. Only about 10% of homozygous offspring survive until weaning. The brain of these mice is larger than that of control mice, with an accumulation of neural progenitors in the periventricular zones. Perturbation induced by *Bax/Bak* loss leads to behavior defects, including being unresponsive to auditory stimuli (Lindsten et al., 2000).

Interestingly, interdigital webs and imperforate vagina were observed in *bax/bak* DKO but not in *caspase-3-* or *caspase-9-* null mice (Lindsten et al., 2000). These diverging phenotypes presumably underscore the role of *Bax* and *Bak* roles in the other types of PCD (Kawai et al., 2009; Karch et al., 2013, 2017).

Although ventricular obstruction was observed due to an abnormal increase of neuron number in *bax/bak* DKO, these mice did not exhibit exencephaly, unlike *caspase-3-* or *caspase-9-* null mice. This difference between these mice may be induced by *Bok*, a multi-motif *Bcl-2* homolog whose functions remain poorly understood.

Single invalidation of *Bok* does not lead to any abnormalities like previously observed in *Bak* KO mice (Ke et al., 2012). DKO of *Bak* and *Bok* induces problems neither in the embryonic stage nor in adult life. DKO of *Bax* and *Bok* does not render more severe phenotypes observed previously in the *bax* KO mice, except for an increase in the ovarian follicle (Ke et al., 2013).

Triple KO of these three proteins gives a more severe phenotype than double invalidation of *bax* and *bak*. The majority of triple KO embryos die before birth at E18. Ninety-nine percent of born mice present severe defects originating from midline fusion perturbation. That is evidence that *Bok* can be partly compensated for the roles of *Bax* and *Bak* during the development (Ke et al., 2018).

3.6.2.2. *Anti-apoptotic proteins*

Although *Bcl-2* is present during embryogenesis, *bcl2* invalidation does not seem to affect the embryonic development in mice (Nakayama et al., 1993; Veis et al., 1993; Michaelidis et al., 1996). *Bcl-2*^{-/-} mice are born normally, but the abnormalities start to develop in the post-natal stage. These mice suffer dramatic consequences on the hematopoietic and immune system: lymphocyte ablation in bone marrow at four weeks of life, hematopoietic cell death increase in their thymus and spleen (Nakayama et al., 1993; Veis et al., 1993). They are died early due to kidney failure developing from polycystic kidney conditions (Veis et al., 1993). Regarding the nervous system, at birth, homozygous *Bcl-2* lacking mice present a functional nervous system with normal morphology. Nonetheless, in the early postnatal stage (postnatal day 9), a progressive degeneration was observed in the population of facial motoneuron and the peripheral neuron in the DRG (Michaelidis et al., 1996).

Conversely, the two anti-apoptotic *Bcl-2* homologs, *Mcl-1* and *Bcl-xL*, appear to hamper apoptotic cell death in neurons and hematopoietic cells during development. Although *mcl1* KO induces pre-implantation embryonic lethality, condition *mcl-1* deletion has revealed its implication in the development of these two populations. In the adult mice, *Mcl-1* deletion leads to perturbation in the lymphoid development and a profound loss of both lymphocytes B and T (Opferman et al., 2003). *Mcl-1* has been found to play important roles in the development of the innate immune system, including maintaining the survival of hematopoietic stem cells, mature neutrophils, and the normal function of macrophages (Opferman et al., 2005; Steimer et al., 2009). Regarding neuron population, *Mcl-1* is critical for the survival of neural progenitors (Arbour et al., 2008). Indeed, condition KO of *mcl-1* in the brain by using promotor *Foxg1* induced embryonic lethality at E16-17 with increased apoptosis of neural progenitor in the ventricular zone (VZ) and subventricular zone (SVZ) (Arbour et al., 2008).

The full KO of *bclx* mice died at embryonic day 13 (E13-13.5). These mice were first generated by Motoyama team. They observed massive apoptosis around the stage of death in the postmitotic neuron of the hindbrain and spinal cord and the hematopoietic cells in the livers (Motoyama et al., 1995). To decipher the reason for embryonic death caused by *Bcl-xL* lacking,

scientists have started to work with conditional KO mice. Although these mice improve our knowledge about the role of Bcl-xL in each particular system, the exact reason why conventional *bclx* KO induces embryonic death remains uncovered. Using promoter MMTV, Wagner and his coworker generated a conditional KO mouse model in which *bcl-x* was lacking in the skin and the hematopoietic system. These mice do not exhibit any abnormalities at birth. However, they develop profound anemia due to severe loss of mature erythrocytes at three months of life (Wagner et al., 2000). Ablation of Bcl-xL in the megakaryocyte, progenitor of the platelets, does not lead to lethality at the embryonic stage. These mice present severe thrombocytopenia at the three-week-old, with platelet counts of only 2% of control mice (Josefsson et al., 2011). Besides, Bcl-xL also regulates the survival of lymphocytes (Ma et al., 1995).

Considering that conditional KO *bclx* in the hematopoietic lineages is not lethal, embryonic lethality in the full KO of *bclx* mice is likely due to the anomalies in the CNS (Motoyama et al., 1995). Notwithstanding, the phenotypes observed from mice with conditional KO in the CNS do not give us a conclusive response. When conditional *bclx* KO is performed under the control of Nestin promoter, homozygous embryos die at the embryonic stage but at the latter stage (E19) than previously described in full KO mice. These embryos present increased apoptosis in the postmitotic motoneuron in the spinal cord (Fogarty et al., 2016). Otherwise, using the *Emx* promoter to delete Bcl-xL only in the developing brain does not lead to embryonic lethality. At birth, the brain of these mice is morphologically and functionally normal. However, a progressive loss of mature neurons occurs in the postnatal stage, resulting in severe microcephaly at P30. In addition, loss of Bcl-xL-dependant neurons leads to behavior perturbations, including motor learning deficits, hyperactivity, and increased risk-taking and self-injurious behaviors (Nakamura et al., 2016).

Altogether, none of the conditional KO mouse models of Bcl-xL hitherto mimic the phenotype of conventional KO mice. That may indicate that the drastic consequence on the total *bclx* KO embryos is the sum of different abnormalities on different systems rather than comes from only single default.

	KO phenotype in mice
Multi-motif pro-apoptotic	
Bax	No lethality. Lymphoid hyperplasia, male infertility (Knudson et al., 1995)
Bak	No defects detected (Lindsten et al., 2000)
Bok	No defects detected (Ke et al., 2013)
Multi-motif anti-apoptotic	
Bcl-2	Post-natal lethality due to polycystic kidneys. Lymphoid degeneration and hair hypopigmentation (Nakayama et al., 1993; Veis et al., 1993)
Bcl-xL	Embryonic lethality at E13.5: massive apoptosis in the CNS and hematopoietic systems (Motoyama et al., 1995)
Mcl-1	Embryonic lethality at E3.5-4 due to peri-implantation defects (Opferman et al., 2005)
Bcl-w	No lethality. Male infertility (Ross et al., 1998)
Bfl-1	No lethality. Dendritic cell apoptosis (Schenk et al., 2017)
Diva/boo (Mouse orthologue of Nrh)	No defects detected (Russell et al., 2002)

Table 2. Summary of mouse phenotype of conventional Bcl-2 protein KO

4. Bcl-xL: where does it stand in the Bcl-2 family?

4.1. Implication of Bcl-xL in the development and pathology

Bcl-xL is a genuinely anti-apoptotic protein of the Bcl-2 family. It exerts the anti-apoptotic activity in a canonical manner at the MOM (Vander Heiden et al., 1997). The *bclx* gene encoding for Bcl-xL contains three exons, in which exon 2 is a target of AS (Boise et al., 1993). Due to the AS, *bclx* expresses under two isoforms: the long-form Bcl-xL (233 amino acids in length) and the short-form Bcl-xS (170 amino acids in length) (Boise et al., 1993).

While Bcl-xL possesses all four conserved BH motifs plus its TM motif, Bcl-xS lacks BH1 and BH2 motifs. This difference in structure converts Bcl-xS into a pro-apoptotic protein. Transient expression of Bcl-xS leads to apoptosis as induced by Bax expression (Lindenboim et al., 2000). Interestingly, *bclxs* mRNA levels are increased in various physiological incidents in which apoptosis is generated, such as brain ischemia and injured carotid artery (Dixon et al., 1997; Igase et al., 1999).

The death-induced activity of Bcl-xS is mediated *via* two mechanisms. On the one hand, it can sequester the anti-apoptotic proteins Bcl-2 and Bcl-xL and inhibits their effect. On the other hand, Bcl-xS can form homodimers, as previously described in Bax and Bak (Lindenboim et al., 2001). However, whether these homodimers can subsequently oligomerize into the pore channel for cytochrome c release remains unclear. Furthermore, Bcl-xL contains a target sequence for cleavage by calpain (after Ala60) and by caspase-3 (after Asp61 and 76) (Fujita et al., 1998; Nakagawa and Yuan, 2000). The cleavage products show pore-forming capacity in the lipid membrane, possibly explaining their pro-apoptotic feature (Basañez et al., 2001). These observations suggest that cells can regulate cell fate by controlling the balance between full-length Bcl-xL and its short forms. Accordingly, manipulating this equilibrium by regulating the *bcl-x* splicing may be a potential therapeutic strategy (Stevens and Oltean, 2019).

In healthy cells, the anti-apoptotic form Bcl-xL is dominantly expressed (Boise et al., 1993). At the cellular level, Bcl-xL locates mainly at the MOM, which is vital for its canonical interaction with pro-apoptotic proteins. However, other cellular locations of Bcl-xL have been identified. The most well-known mitochondria outside localization of Bcl-xL is ER. On this organelle, Bcl-xL prevents the pro-apoptotic Ca^{2+} wave from ER to mitochondria, as detailed in the previous section (White et al., 2005; Monaco et al., 2012).

Bcl-xL acts both as a "death resistance fighter" and a "life sponsor" to favor cell fate toward cell survival. Bcl-xL has been found to promote the activity of F0F1 ATPase in the

mitochondrial inner membrane by reducing H⁺ leak (Alavian et al., 2011). At the ER, it enhances the pro-survival Ca²⁺ oscillations, which promote the activity of Krebs's cycle enzymes. Altogether, Bcl-xL boosts ATP production and biogenesis (Monaco et al., 2012; Rossi et al., 2019).

Bcl-xL is also addressed to more "exotic" locations. A portion of Bcl-xL translocates into the nucleus during the G2/M checkpoint and in some cancer contexts (Schmitt et al., 2007; Choi et al., 2016). This nuclear fraction promotes metastasis of cancer cells independent of Bcl-xL anti-apoptotic function (Choi et al., 2016). Moreover, there is evidence suggesting Bcl-xL localization at the peroxisome. Indeed, Bcl-xL expression inhibits Bak-induced catalase release without modifying Bak peroxisome localization (Hosoi et al., 2017). This suggests a direct interaction between Bcl-xL and Bak at the level of peroxisome. However, further investigation is necessary to confirm this point solidly.

Despite their high similarity in sequence and function, Bcl-xL and Bcl-2 exhibit different cell-lineage specificity. Bcl-xL is widely present in the brain, kidney, and thymus, while Bcl-2 is more expressed in the lung, ovarian, breast, and prostate.

During development, Bcl-xL expression starts at the early blastocyst and reaches its peak during neurogenesis (Krajewska et al., 2002; Metcalfe et al., 2004). Unlike Bcl-2, the Bcl-xL level is maintained at its peak in the CNS during embryogenesis and post-natal stage and remains high through adulthood (Krajewska et al., 2002). In the mature brain, Bcl-xL is the dominant anti-apoptotic protein.

Bcl-xL presence is essential for the survival of neurons. *Bclx* KO leads to mouse embryonic lethality at E13.5 with massive apoptosis in the CNS and hematopoietic system (Motoyama et al., 1995). Later, the role of Bcl-xL in these populations was confirmed by conditional KO using the Cre-loxP system (Wagner et al., 2000; Josefsson et al., 2011; Fogarty et al., 2016; Nakamura et al., 2016).

In the CNS, Bcl-xL protects the post-mitotic neuron population but not the progenitors (Motoyama et al., 1995; Nakamura et al., 2016). Conditional KO of Bcl-xL in neural progenitors using Nestin promoter also induced embryonic death, which is delayed compared to the total KO (Fogarty et al., 2016). When the KO is limited in the developing brain using *Emx1* (a specific marker of cerebral cortex pyramidal neurons), the homozygous mice are born with normal morphology but suffer neural loss since birth (Nakamura et al., 2016). The

difference between these mice may be due to the timeline gap of neurogenesis waves between the spinal cord and cerebral cortex.

Bcl-xL also protects neurons against apoptosis during injury. After brain ischemia, the Bcl-xL level rapidly increases (Wu et al., 2003). Bcl-xL introduction into hypoxic PC12 culture rescues ROS-related cell death (Shimizu et al., 1995). Furthermore, injection of fusion Bcl-xL-protein transduction protein of TAT peptide decreases up to 40% infarction in the brain of ischemic mice (Cao et al., 2002).

In contrast to its protective role, Bcl-xL can also induce neuronal death in response to neurotoxins. As mentioned above, Bcl-xL can be processed by proteases to form pro-apoptotic Δ N-Bcl-xL. During cerebral ischemia, Δ N-Bcl-xL generated by caspase-3 causes hippocampal CA1 neuron death, which is agonized by Bcl-2/Bcl-xL inhibitor ABT-737 (Ofengeim et al., 2012). Glutamate excitotoxicity also induces Δ N-Bcl-xL accumulation, which leads to the mitochondrial potential drop (Park et al., 2017).

In addition, Bcl-xL plays other roles than only neuroprotective in the neurons. Bcl-xL is required for neurite outgrowth and branching, which is crucial for the normal development of the nervous system (Park et al., 2015). Bcl-xL also promotes the formation of synapses between neurons (Li et al., 2008a). In the mature synapse, Bcl-xL participates in the SV endocytosis (Li et al., 2013).

Strikingly, during these studies on non-apoptotic roles of Bcl-xL performed by Jonas group, inhibition of Bcl-xL by pharmacological inhibitors or genetic loss does not lead to immediate neuron death (Li et al., 2008a, 2013; Park et al., 2015). Exposure to ABT-737 up to a concentration of 10 μ M does not induce cell death in the mature neuron but aberrance in the SV endocytosis (Li et al., 2013). The depletion of Bcl-xL by siRNA in the developing hippocampal culture does not lead to apoptosis even after 2.5 weeks. This lack of Bcl-xL in the developing neuron causes instead abnormal neurite outgrowth (Li et al., 2008a). These observations suggest that neuronal death may not directly result from Bcl-xL lack but the secondary consequence of neural development and maturation failure.

Bcl-xL is implicated in various pathological contexts: the most highlighted are cancer and neurodegenerative diseases.

Bcl-xL upregulation has been detected in multiple cancers, including breast cancer (España et al., 2004), hepatocellular carcinoma (Watanabe et al., 2002), renal carcinoma (Gobé et al.,

2002), prostate cancer (Lebedeva et al., 2003), and pancreatic cancer (Ghaneh et al., 2002). Increased Bcl-xL level is correlated with tumor progression and chemotherapeutic resistance (Campbell and Tait, 2018).

Like other Bcl-2 anti-apoptotic proteins, Bcl-xL has been thought to contribute to cancer development mainly through apoptosis evasion (Campbell and Tait, 2018). Cell death can be triggered by regulating the ratio of Bcl-2 proteins toward pro-apoptotic members. A class of therapeutic agents has been developed based on this concept, named BH3-mimetics. These molecules target and neutralize the Bcl-2 anti-apoptotic proteins, including Bcl-xL. They liberate and activate pro-apoptotic proteins Bax/Bak.

A growing body of evidence reveals the non-apoptosis-related roles of Bcl-xL in cancers. Bcl-xL promotes tumor invasiveness independent of apoptosis inhibition or tumor growth (Ho et al., 2010; Choi et al., 2016; Trisciuglio et al., 2017). Interestingly, Bcl-xL KO decreases the tumor invasiveness while Bcl-xL overexpression links to tumor burden increase (Naik et al., 1996; Hager et al., 2009). This role is likely related to a pool of Bcl-xL in the nucleus and does not implicate the interaction Bcl-xL – Bax/Bak (Choi et al., 2016).

Neuronal loss underlies neurodegenerative disorders (NDs), including amyotrophic lateral sclerosis (ALS), Parkinson's, Alzheimer's, and Huntington's disease. Cell death usually begins in specific neural populations, then expands to different regions of the CNS, and finally shuts down entirely vital functions (Mattson, 2000). Caspase-dependent cell death plays a key role in the pathology of these diseases (Dugger and Dickson, 2017; Moujalled et al., 2021). As mentioned above, Bcl-xL is the main anti-apoptotic protein present in the mature nervous system, while Bcl-2 expresses mainly in the microglia of the adult brain (González-García et al., 1995; Krajewska et al., 2002). Consequently, Bcl-xL is thought to be a critical factor in the development of NDs.

Unexpectedly, the literature on the implication of Bcl-xL in the ND is rather limited.

In the HD, Bcl-xL levels do not change in the brain of the HB mouse model (Hansson et al., 2001; Zhang et al., 2003). Only one study links Bcl-xL with HTT protein turnover. In this study, Khosnan and co-workers found that DNA damage can induce the HTT proteolysis *via* I κ B kinase (IKK), both full-length and mutant polyQ forms. Activation of IKK β by DNA damage increases Bcl-xL phosphorylation and decreases Bcl-xL level, subsequently leading to the activation of caspase-3 that cleaves the protein Htt (Khosnan et al., 2009).

Bcl-xL is essential for the normal development of the mouse substantia nigra. Lack of Bcl-xL induced catecholaminergic neuron reduction. However, it does not lead to total depletion of the catecholaminergic cell population, suggesting that Bcl-xL plays a critical role in the survival of a selective population (Savitt et al., 2005). Furthermore, Bcl-xL rescues, in vitro and in vivo, the neuron treated with 6-Hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), two agents utilized in modeling Parkinson's disease in mice (Jordán et al., 2004; Dietz et al., 2008).

Interestingly, presenilin-1 and -2, two proteins involved in Alzheimer's disease, sequester Bcl-xL to non-mitochondrial localization, leaving the mitochondria "unprotected" from apoptotic stimuli (Passer et al., 1999).

All these studies mentioned here focused only on the apoptosis-related role of Bcl-xL, in which the principal hypothesis was that abundant apoptosis was induced by Bcl-xL expression level drop. It is somewhat an old simple idea about how this protein works. With emerging knowledge about the non-apoptotic roles of Bcl-xL in neurons and the complexity of its functions and regulation, its involvement in the nervous system development and the NDs should be revisited.

Accordingly, Bcl-xL is an interesting research target, and more profound knowledge about its functions, regulation, and interaction partners would benefit both fundamental research and therapeutics.

4.2. Aim of the project

As discussed throughout my bibliography review, the interactome of the Bcl-2 protein family has emerged, which linked it to non-canonical roles beyond apoptosis. Bcl-xL is an apoptotic protein that is ubiquitously expressed and vital for the development of the CNS (Motoyama et al., 1995). Observations from KO mouse models initially suggested its critical role in controlling neural death: its loss drives the neuron into death fate. Notwithstanding, further studies challenge this traditional point of view. Indeed, Bcl-xL loss in the neuron culture does not result in immediate death but abnormalities in the neural development and function (Li et al., 2008a, 2013; Park et al., 2015). Hence, the cell death previously observed in KO mice is possibly a tardive and indirect consequence of improper maturation.

Bcl-xL has a dual localization: mitochondrial and ER (Eno et al., 2012). The mitochondrial pool of Bcl-xL is addressed to its protective role against apoptosis *via* neutralizing apoptotic protein Bax and Bak. ER pool of Bcl-xL is linked to Ca^{2+} homeostasis. In addition, Bcl-xL exerts its protective activity remotely from ER. Through interaction with IP₃R, Bcl-xL prevents the pro-apoptotic Ca^{2+} wave from ER to mitochondria, therefore protecting the MOM integrity (Rosa et al., 2022). A recent study by our team shed light on the complementary protective mechanism of Bcl-xL in the ER. It appeared that inhibition of IP₃R-mediated Ca^{2+} by Bcl-xL also protects the cell from ER stress by preventing ER Ca^{2+} depletion (Jabbour et al., BioRxiv).

It is potential that Bcl-xL also regulates Ca^{2+} -related cellular processes, such as migration, exocytosis, and protein secretion. In a previous study by our team, we demonstrated an exclusive non-apoptotic role of the protein Nrz, a zebrafish ortholog of the anti-apoptotic protein, during the development. By regulating IP₃R-related Ca^{2+} release, Nrz controls the cytoskeletal network of the yolk cell for proper epiboly (Popgeorgiev et al., 2011; Bonneau et al., 2014).

Based on the literature and the results we accumulated in the team, we started a project with an ambitious objective: to distinguish the role of mitochondrial and ER pools of Bcl-xL in mammalian development. To answer this question, our team has generated two knock-in mouse models in which Bcl-xL is expressed exclusively either at the mitochondria or at the ER. Far from our expectation, the mice with Bcl-xL exclusively at the ER (hereafter called ER-xL mice) exhibit abundant cleaved caspase-3 in the spinal cord white matter at E13.5 while much lesser in the grey matter.

From this primary, we hypothesized that Bcl-xL lack at the mitochondria led to local activation of caspase-3, which might not be related to apoptosis. Hence, my Ph.D. project aims to answer three questions:

- 1- Does mitochondrial Bcl-xL absence induce a caspase-3 local activation?
- 2- What are the consequences of local active caspase-3 in neuron development?
- 3- What is the mechanism of the caspase-3 local activation?

Firstly, we investigated the active caspase-3 status in the brain of ER-xL mice. We confirmed that active caspase-3 is present more in the corpus callosum than the cerebral cortex. Using the microfluidic system, we found that ER-xL cortical neurons exhibit active caspase-3 signals in the axons more in the soma. In addition, we did not detect an increase in apoptosis in mutant neurons compared to WT. Afterward, we studied the consequences of active caspase-3 on axon and synapse development. We demonstrated that caspase-3 activation in the axon defects the axon branching and synapse formation. This observation may explain the phenotype we observed in vivo of ER-xL mice: they are born without visible anomalies but fail to nurse and die rapidly within 12 hours. We then thought that the lack of Bcl-xL at the mitochondria might perturb their network and functions, which led to caspase-3 activation. Hence we assessed the morphology, membrane potential, and motility of axonal mitochondria.

Interestingly, mutant neurons have mitochondria in the axon that are more fragmented, and their membrane potential is lower. We then assessed the fusion and fission machinery. Interestingly, we found that ER-xL losses interaction with Drp1, a key protein of mitochondria fission. Treatment of Drp1 inhibitor, m-divi, diminishes the caspase-3 activation in the axon of mutant neurons.

Our study suggests that the mitochondrial pool of Bcl-xL is necessary to confine the local caspase-3 activation to appropriate physiological intensity in the developing CNS. These findings bring a novel point of view on the regulatory role of Bcl-2 proteins in the physiological and pathological non-lethal caspase-3.

RESULTS

Mitochondrial Bcl-xL controls non-lethal caspase activation to promote brain synaptogenesis

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ABSTRACT

Non-lethal caspase activation (NLCA) has previously been linked to neurodevelopmental processes, including axon guidance, synapse formation, and axon pruning. However, how neurons spatio-temporally process and control NLCA remains elusive. Here, we focused on Bcl-xL, a Bcl-2 homolog, which controls caspases activation through Bax/Bak inhibition at the mitochondrial outer membrane (MOM).

We generated a genetically-modified mouse model, referred to as ER-xL, in which Bcl-xL was targeted exclusively at the endoplasmic reticulum (ER), being absent at MOM. Compared to *Bclx* knockout mice, which died at E13.5, ER-xL mice survived during embryonic development but died rapidly after birth due to altered feeding and swallowing behavior. Immunocytochemistry experiments detected and enhanced caspase-3 activity in the brain and the spinal cord white matter but not the grey matter. Interestingly, primary cultures of ER-xL cortical neurons did not reveal increased cell death levels, suggesting that the observed caspase-3 activation was independent of apoptosis. In effect, ER-xL neurons showed increased caspase-3 activity in the neurites, which resulted in impaired axonal arborization and deficient synaptogenesis. Furthermore, mitochondria in the ER-xL axon were less fused and more motile than in the WT axon. Notably, treatment of ER-xL axons with the Drp-1 inhibitor, m-divi, restored baseline caspase-3 activity.

Altogether, our findings suggest that during development, the mitochondrial pool of Bcl-xL finely tunes caspase-3 through Drp-1-dependent mitochondrial fission, which is critical to synaptogenesis and neural network design.

INTRODUCTION

Cysteine-dependent aspartate-directed proteases (caspases) are a family of intracellular proteases playing essential roles in inflammation and cell death in metazoans (Wolf and Green, 1999; Van Opdenbosch and Lamkanfi, 2019). Caspases involved in apoptosis are classified into two groups: the initiators (caspase-8, -9, and -10) and the executioners (caspase-3, -6, and -7). Once activated, the initiators will activate the executioners, cleaving critical cellular proteins leading to cell death (Parrish et al., 2013). By acting as apoptosis executioners, these enzymes control the size of cell populations and participate in tissue morphogenesis during embryonic development, including in the brain and the spinal cord. Lack of caspases activity is linked to neural malformations and defects (Liu et al., 2018; Spellicy et al., 2018; Zhou et al., 2018).

In recent years, new studies have demonstrated that caspase activation is not always associated with apoptosis (D'Amelio et al., 2010; Brokatzky et al., 2019). For instance, treating cancer cells with Bcl-2 inhibitors, namely BH3-mimetics, led to a limited fraction of permeabilized mitochondria: a process called minority MOMP (miMOMP) (Ichim et al., 2015). MiMOMP led to non-lethal caspase activation (NLCA) and subsequently increased genomic instability, cell migration, and invasiveness (Ichim et al., 2015; Berthenet et al., 2020). Intriguingly, sublethal caspase levels have also been found to promote neural development processes, including axon branching and synapse formation, but the control of NLCA remains elusive (D'Amelio et al., 2010; Unsain and Barker, 2015; Mukherjee and Williams, 2017).

Bcl-2 proteins have been widely studied for their role in the regulation of apoptosis. These proteins control the activation of caspase-3 by regulating the release of apoptogenic factors from the mitochondria to the cytosol in a process called mitochondrial outer membrane permeabilization (MOMP) (Gross et al., 1999; Chipuk et al., 2010). Notably, MOMP is initiated through the oligomerization of pro-apoptotic effectors Bax and Bak and is repressed by the anti-apoptotic Bcl-2 proteins, which bind and inhibit Bax/Bak oligomerization. The imbalance

between cell death and survival is involved in a number of pathologies, including developmental defects, degenerative diseases, and cancers (Mattson, 2000; Delbridge et al., 2016; Opferman and Kothari, 2018).

The Bcl-xL protein is a Bcl-2 homolog playing a key role in embryonic development. In the mammalian embryo, its expression is ubiquitous, and particularly high levels have been measured in the nervous system (Lindsten et al., 2005). Invalidation of the *Bclx* gene in mice causes early embryonic death (E13.5), which has been attributed to increased apoptosis in the nervous and hematopoietic systems of *Bclx* knockout (KO) mice (Motoyama et al., 1995). In addition, although Bcl-xL has a mitochondrial localization, it is also found in the endoplasmic reticulum (ER), where it participates in the regulation of IP3-dependent intracellular calcium trafficking (Morris et al., 2021; Rosa et al., 2022).

To investigate the impact of Bcl-xL intracellular partitioning during mice development, we used a knock-in (KI) strategy in mice to selectively direct the Bcl-xL protein to the ER.

Here, we report that the absence of Bcl-xL in the mitochondria, while the protein is maintained in the ER, was sufficient to sustain embryonic development and birth. However, homozygous mutant mice (referred to as ER-xL) died at post-natal day 1 (P1), displaying swallowing and feeding defects. Histochemical analyses performed at embryonic stage E13.5 revealed an increase in caspase-3 activity in the spinal cord, the brain stem, and the cortex. Caspase-3 staining was intense and homogeneous in the white matter but sparse in the grey matter, suggesting an impact on neurites but not on the cell body. Culture of primary ER-xL neurons derived from the cortex confirmed that the loss of mitochondrial Bcl-xL leads to caspase-3 activation in the neurites without a significant increase in apoptosis.

Collectively, our data indicate that such caspase-3 activation impacts neurite outgrowth and synaptic connections. These data suggest that Bcl-xL controls local activation of caspase-3 in

the neurites to ensure neuronal development. Hence, our findings open new avenues regarding the role of Bcl-xL in the development and neurodegenerative diseases.

RESULTS

Generation of ER-Bcl-xL mice. Bcl-xL actively cycles between the cytosol and the mitochondria, and was also shown to locate at ER membranes. To assess the specific contribution of the ER-located Bcl-xL to embryonic development, we generated a knock-in (KI) mouse strain (herein referred to as ER-xL), expressing a recombinant Bcl-xL protein exclusively in the ER. Based on the CRE/LOXP technology, we targeted the exon 3 (E3) in the wild type (WT) allele. *Bclx* E3 encodes for the C-terminus transmembrane (TM) motif of Bcl-xL. We replaced this sequence with an engineered E3 containing the TM motif of the ER-based Cytochrome B5 (CB5) protein. Cre-recombination induced WT E3 excision and CB5 mutant expression (Figure 1 A,B). Expression of recombinant ER-Bcl-xL was verified in lung, liver and brain tissues (Figure 1C). Furthermore, the expected subcellular localization of WT and ER-Bcl-xL was verified in brain tissue using subcellular fractionation (Figure 1 D,E).

Characterization of the phenotype of ER-xL mice. Though *Bclx* KO was reported to lead to embryonic death at E13.5 (Motoyama et al., 1995), ER-xL mice survived beyond that stage and were born according to Mendelian rules (Figure S1A). Interestingly, ER-xL mice were significantly smaller than WT or heterozygous (HET) littermates (Figures 2 A & S1 B,C). ER-xL mice lacked visible milk pockets, and when artificially fed, presented incoherent sucking and swallowing reflexes and were unable to survive beyond 24 hours (Figure 2B).

To further investigate this phenotype, we conducted a histological study focusing on the nervous system using cleaved caspase-3 as a marker of apoptosis. We observed increased caspase-3 signaling in the cortex (Figure 2 C-G) but also in the hindbrain and the spinal cord (Figure S2).

Interestingly, this signal was mainly detected in the white matter, but not the grey matter where the cell bodies of cortical neurons are located, suggesting that the increased caspase-3 signal is not related to apoptosis (Figure 2 F-G & S2 B,E). To test this hypothesis, we isolated and

cultured primary cortical neurons for 5-7 days (Figure 3 A). Under these conditions, we did not observe a higher rate of pyknotic nuclei in neurons from ER-xL mice, compared to WT (Figure S3). In contrast, primary ER-xL neurons exhibited positive caspase-3 signal in the neurites, but not in the cell body (Figure 3 B-D).

These observations raise issues regarding the consequences of this elevated caspase-3 activity and the mechanisms that allow caspase-3 activation in neurites, independent of cell death.

Analysis of neurite architecture. Next, we investigated the impact of ER-Bcl-xL expression on axon morphology. We isolated embryonic cortex neurons and performed electroporation with a Venus-expressing plasmid. Cortical neuron dissociation was performed at E15.5 and axon morphology was analyzed. The overall axon length between ER-xL and WT primary neurons was similar (Figure S4 A). However, we detected a decrease in axon arborescence in ER-xL neurons compared to WT mice (Figure 4 A,B & S4B). Furthermore, although the density of dendritic spines was unchanged (Figure S4C), we observed notable changes in their morphology, with a decrease in the number of mushroom type dendritic spines in ER-xL primary neurons and an increase in filopodial spines (Figure 4 C-E).

Finally, we analyzed the effect of the caspase-3 inhibitor Z-DEVD-FMK on the axon morphology of ER-XL neurons. Treatment of neurons with 10 μ M of Z-DEVD-FMK restored both axon arborization and dendritic spine organization (Figure 4 F-G). Hence, these observations suggest that the local increase in caspase-3 activity in ER-xL axons drives morphological changes in neurite architecture.

Analysis of synaptogenesis capacities. The above observations suggest that ER-xL mice may display alterations in neural network architecture as a result of deficient synaptogenesis. In order to assess this point, we implemented a microfluidic approach to study pre- and post-synaptic branching. In these experiments, neurons were placed in two compartments (referred

to as pre- and post-synaptic) connected by micro-channels to a synaptic chamber, in order to follow neurite elongation and synapse formation.

First, we seeded both compartments with the same type of neurons (pre-WT/post-WT and pre-ER-xL/post-ER-xL). A caspase-3 signal could clearly be observed when compartments were seeded with ER-xL but not WT neurons, confirming the results from primary cultures (Figure 5 A-D). Synaptic connections were analyzed with antibodies against Synaptophysin and PSD95 used as markers of pre- and post-synaptic compartments, respectively. These latter experiments highlighted a deficit in synaptogenesis when performed with ER-xL neurons, compared to WT (Figure 5 E-F).

Second, synaptogenesis was monitored when compartments were cross seeded with different cell types (ER-xL or WT). As shown in Figure 6, synaptogenesis was only impacted when ER-xL neurons were present in the pre-synaptic compartment, irrespective of the cell type in the post-synaptic compartment. These findings suggest that defects at the level of axon terminals may be responsible for the synaptogenesis alterations observed.

Analysis of mitochondria dynamics in cortical axons. Activation of caspase-3 is mostly, although not exclusively, dependent on mitochondrial outer membrane permeabilization (MOMP). Thus, during apoptosis, activation of the caspase cascade, including downstream caspase-3, follows a decrease in MOM and the subsequent release of cytochrome c into the cytosol. This prompted us to examine the status of mitochondria in ER-xL and WT axons.

First, we measured the mitochondrial membrane potential ($\Delta\Psi_m$) using the tetramethylrhodamine ethyl ester (TMRE). $\Delta\Psi_m$ was significantly lower in the mitochondria of ER-xL axons, compared to WT mice (Figure 7 A). This decrease was not due to an overall decrease in the area or the amount of axon mitochondria (Figure 7 B-C). However, we found that the level of mitochondrial fission was higher in ER-xL axons, compared to WT (Figure 7

D-E). In addition, when we measured their velocity we found that ER-xL mitochondrial axons were more mobile compared to mitochondria in WT axons (Fig. 7 F-G).

Previous studies unveiled Bcl-xL/dynamin-related protein 1 (Drp-1) complexes in neurons (Li et al., 2008b, 2013). The dynamin-like GTPase Drp-1 is recruited to the MOM where it drives mitochondria fission *via* GTP-dependent constriction and fosters cytochrome c release during apoptosis (Estaquier and Arnoult, 2007; Prudent et al., 2015b; Banerjee et al., 2022). This prompted us to investigate the interaction between Bcl-xL and Drp-1. We performed co-immunoprecipitation experiments using WT Bcl-xL or ER-addressed Bcl-xL as baits. Compared to native Bcl-xL, ER-Bcl-xL interacted only weakly with Drp-1, suggesting that the Bcl-xL/Drp-1 complex was strongly decreased in ER-xL neurons (Figure 8 A). In this respect, it is worth be noted that Drp-1 total amount was unchanged in Er-xL neurons, compared to WT (Figure 8 B). To further investigate this finding, we analyzed the effect of the Drp-1 inhibitor m-divi on caspase-3 activity in ER-xL axons. As shown in Figure 8 C-E, the caspase-3 signal was suppressed in ER-xL axons, following m-divi treatment, supporting the notion that caspase-3 activation in ER-xL axons is Drp-1-dependent.

DISCUSSION

Loss of mitochondrial Bcl-xL does not cause embryonic lethality. Here, we report that lack of mitochondrial Bcl-xL generated by a knock-in strategy in mice does not lead to embryonic death as previously described in *Bclx* knockout mice (Motoyama et al., 1995). ER-xL embryos do not exhibit detectable organ alterations as shown by histological analyses. ER-xL newborns survive up to 12 hours with apparent normal mobility.

The delayed lethality compared to *Bclx* knockout mice may result from a “milder intensity” of cell death in ER-xL individuals. Indeed, in ER-xL embryos (E13.5), an increase in caspase-3 signals in the spinal cord and the brainstem could be detected, but not in the liver. This is in sharp contrast with *Bclx* knockout mice, where massive apoptotic bodies were reported in the central nervous system (CNS) and the hematopoietic cell population in the liver at the same embryonic stage (Motoyama et al., 1995).

Role of ER Bcl-xL in development. The observed phenotype of ER-xL mice, also suggests that the maintenance of the ER pool of Bcl-xL prevents premature embryonic lethality, suggesting that ER-Bcl-xL plays a protective role, presumably through ER Ca^{2+} trafficking in neurons and hematopoietic cells. Indeed, ER stress has been reported to contribute to neurogenesis, and cortex development (Mimura et al., 2008; Cho et al., 2009; Laguesse et al., 2015). It should also be noted that a peak in the unfolded protein response (UPR) occurs before embryonic death E13.5 in the mouse embryo (Motoyama et al., 1995; Frank et al., 2010). In this respect, we found that mouse embryonic fibroblasts derived from ER-xL mice are more vulnerable to mitochondrial stress stimuli, but more resistant to ER stress inducers (Jabbour et al., 2021). Thus, ER-Bcl-xL is conceivably able to regulate developmental UPR and block ER-stress-dependent neuronal cell death during early development.

Mitochondrial Bcl-xL and axonal caspase-3 activation. Histological analyses revealed that active caspase-3 signaling is higher in the white matter than the grey matter in both the spinal cord and the cortex of ER-xL embryos. Primary cultures of cortical neurons confirmed that such caspase-3 activation was independent of apoptosis. Moreover, microfluidic studies carried out on ER-xL neurons, revealed that activated caspase-3 was predominantly found in axons.

Similar local caspase-3 activation has been described in the developing CNS (Campbell and Holt, 2003; Cusack et al., 2013; Guo et al., 2016), caspase-3 having been reported to play a role in axon branching, as well as axon and spine pruning.

Here, local caspase-3 activation was also detected in WT neurons *in vitro*. However, the marked increase in caspase-3 activity observed in ER-xL neurons is presumably a causal event regarding neural development abnormalities, leading to premature death shortly after birth.

Local caspase-3 activation impacts axon branching and synaptogenesis. We observed that active caspase-3 *in vitro* can preferentially be found in branching points and contact sites between neurons. Thus, we hypothesized that aberrant local caspase-3 activation might result in abnormal axon development and synapse formation.

Indeed, morphological analyses performed on ER-xL cortical neurons showed axon branch reduction without any impact on axon length. Treatment with z-DEVD-fmk confirmed that the observed decrease in axon branch was due to caspase-3 activation in axons. Paradoxically, transient caspase-3 activation was previously reported to be required for axon branch formation (Campbell and Okamoto, 2013). In the same line, other studies highlighted the role of caspase-3 in axon pruning (Nikolaev et al., 2009; Cusack et al., 2013).

Collectively, these data suggest that while baseline caspase-3 activity is critical for the design of axon arborescence, the levels of caspase-3 activity in ER-xL neurons are out of range resulting in deleterious effects on synaptogenesis.

ER-xL neurons exhibit abnormalities in synapse formation and maturation. Microfluidic analyses suggested that these synaptic alterations depended on the pre-synaptic compartment. In this respect, it should be reminded that Bcl-xL was reported to control vesicular trafficking in axon terminals, such control might thus be altered in the ER-xL context, impacting synaptogenesis (Li et al., 2013).

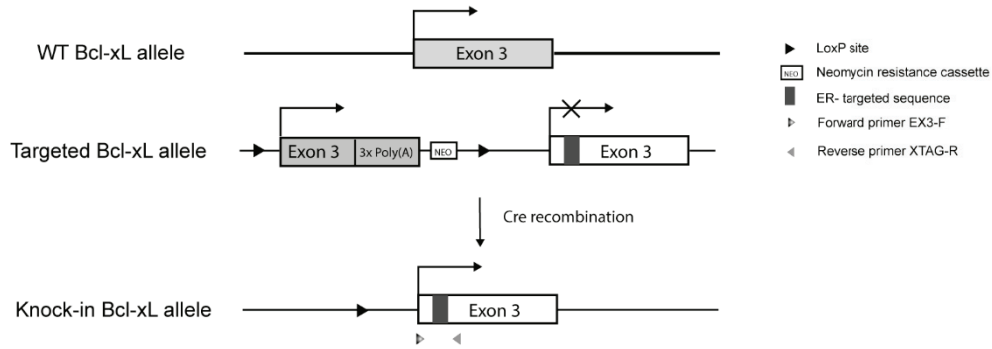
We further investigated synapse maturation by assessing the morphology of dendritic spines, a structure on the dendrites corresponding to the postsynaptic moiety. ER-xL neurons presented a decreased number of mushroom-like spines, which may correspond to mature glutamatergic synapses. This would be worth confirming using immunolabelling experiments.

Mitochondrial status. Our data also indicate that the status of mitochondria is altered in axons from cortical ER-xL neurons with changes reminiscent of apoptosis, such as increased Drp-1-dependent fragmentation, increased motility and drop of $\Delta\Psi_m$. They also suggest that caspase-3 may be activated as a consequence of these mitochondrial changes. However, such a mitochondria-dependent activation is likely to be strictly controlled over time and space since it is limited to the neurites without detectable apoptosis. Consistently, the contribution of ER-Bcl-xL might be critical. Finally, our observations are reminiscent of incomplete MOMP (iMOMP) and subsequent sublethal caspase-3 activation (Cao et al., 2022).

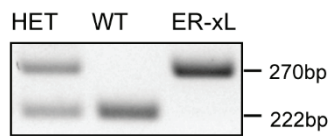
Overall, our data support the idea that Bcl-xL, controls iMOMP in axons by acting on the mitochondrial network in a Drp-1-dependent manner. Although a number of issues remain to be addressed, in particular figuring out why mitochondria are not impacted in the soma of ER-xL neurons, the present report sheds new light on the role of Bcl-2 family proteins in the developing CNS and may open novel avenues regarding neurodegenerative diseases.

FIGURES

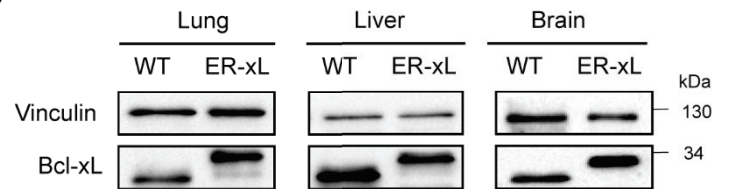
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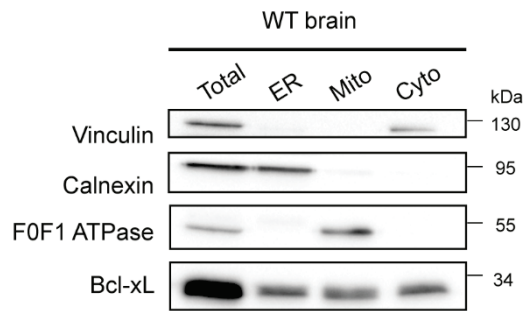
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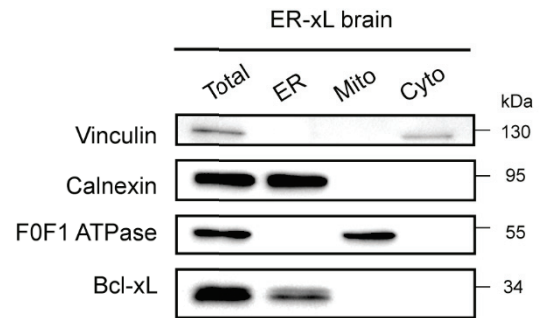


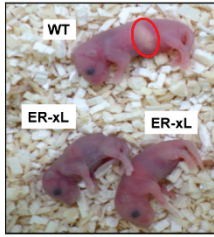
Figure 1. Generation and characterization of ER-xL mice

(A) Schematic representation of the gene targeting strategy: an engineered vector containing a WT Bcl-xL exon 3 (E3) flanked between two LoxP sites, and a modified E3 targeting ER was inserted to replace WT Bcl-xL E3 in floxed mice. Cre recombination induced WT E3 and allowed ER-targeted E3 expression. The sites of EX3-F and XTAG-R for PCR genotyping are shown.

(B) PCR genotyping of WT, ER-xL, and heterozygous mice. The 270-bp band indicates the presence of the ER-targeted E3 allele, while the 222-bp band indicates the presence of WT E3.

(C) Expression of the Bcl-xL protein in the lung, liver, and brain tissues of WT and ER-xL mice. Western blot analysis was performed with indicated antibodies. Vinculin was used as a loading control.

(D-E) Expression of the Bcl-xL protein in the subcellular fractions of ER, mitochondria, and cytosol from post-natal P1 brain of WT (D) and ER-xL (E) mice. Western blot analysis was performed with indicated antibodies. Vinculin was used as a cytosolic marker, calnexin as an ER marker, and F0F1 ATPase as a mitochondrial marker.

A**B**

		Died before 12h	Died at 12-24h	Survival to 24h	Total
Normal condition	WT	0 (0%)	0 (0%)	10 (100%)	10
	ER-xL	5 (71.4%)	2 (28.6%)	0 (0%)	7
Artificial feeding	WT	0 (0%)	0 (0%)	10 (100%)	10
	ER-xL	3 (42.8%)	3 (42.8%)	1 (14.3%)	7

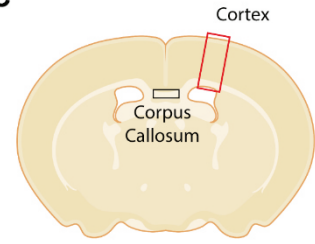
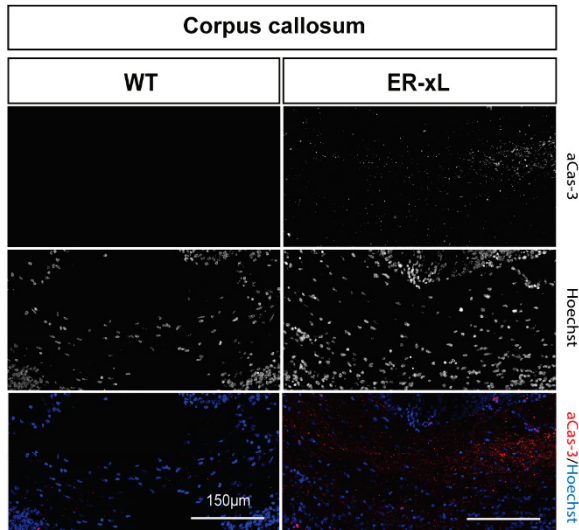
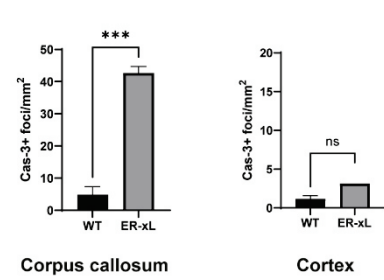
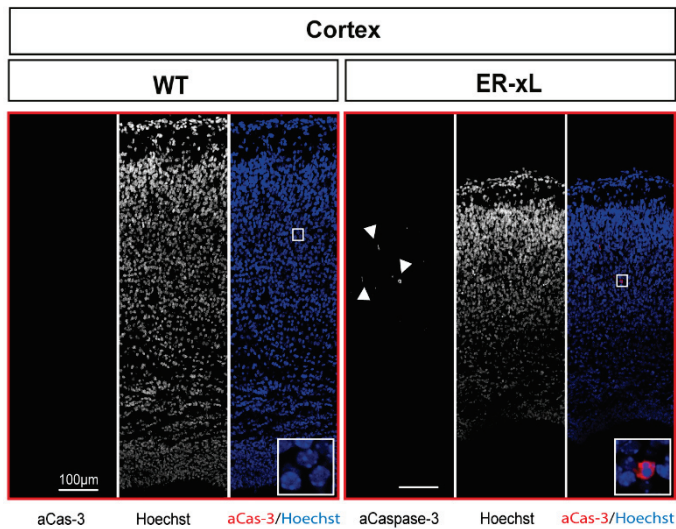
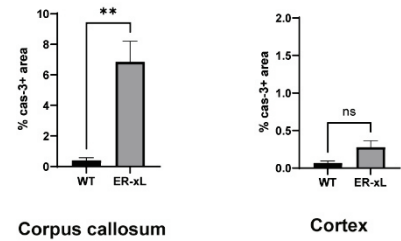
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Figure 2. Characterization of ER-xL mouse phenotype

(A) Image of newborn WT and ER-xL mice (phenotype of each mouse is indicated). The milk pocket is visible in the WT newborn (red circle).

(B) Table showing number of dead and live ER-xL, HET, and WT newborns over the first 24 hours with normal conditions (with mother mouse) or with artificial feeding.

(C) Illustration of the relative position in the whole coronal brain section of high magnification images in (D-E).

(D-E) Representative high-magnification images of the corpus callosum (D) and cortex (E). Immunofluorescence was performed with active caspase-3 (aCas-3). Arrowheads point to active caspase-3-positive cell bodies. Nuclei were marked with Hoechst.

(F) Quantification of active caspase-3 foci density in the WT and ER-xL corpus callosum and cortex. Data show average value \pm SEM. Data are from three independent experiments. Student t-test. $N_{WT} = 3$, $N_{ER-xL} = 3$.

(G) Percentage of surface area with active caspase-3 in the WT and ER-xL corpus callosum and cortex. Data show average value \pm SEM. Data are from three independent experiments. Student t-test. $N_{WT} = 3$, $N_{ER-xL} = 3$.

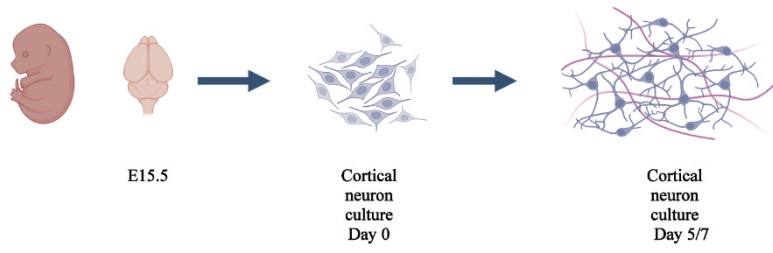
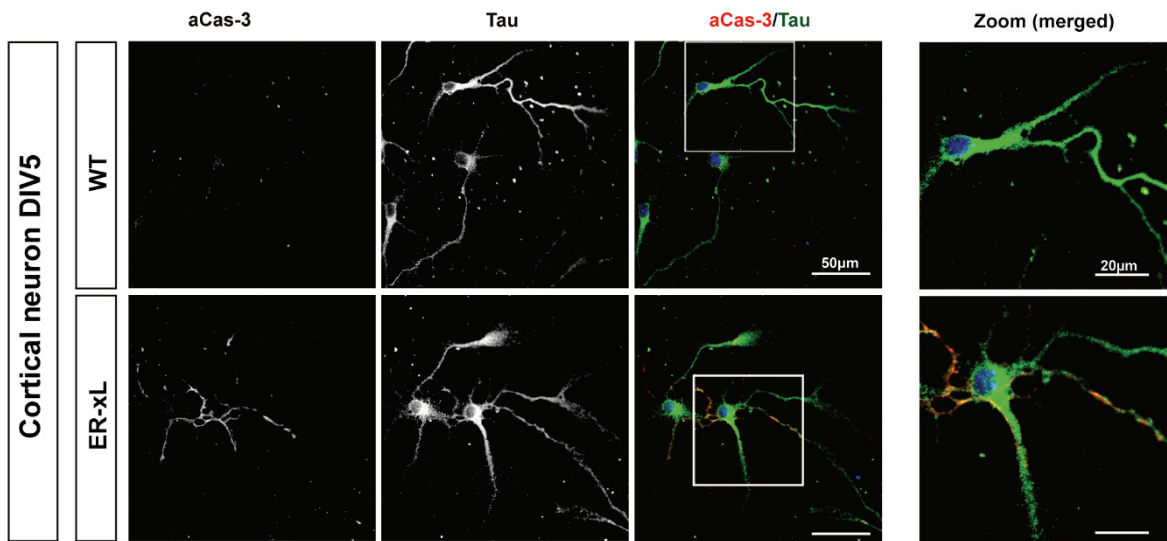
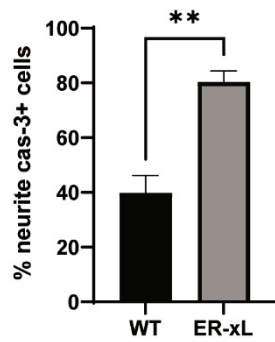
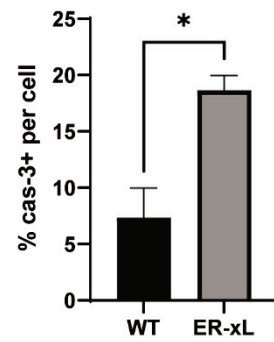
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Figure 3. Loss of mitochondrial Bcl-xL induces caspase-3 activation in the neurites of cortical neurons in culture

(A) Schematic diagram of the cortical neuron isolation procedure. Cortical neurons were isolated from the cerebral cortex of the E15.5 embryo and maintained in culture for 5-7 days in vitro (DIV 5-7).

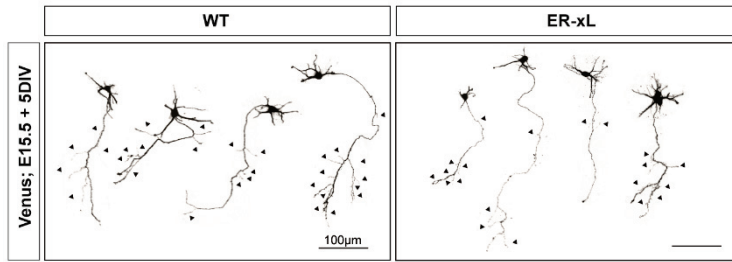
(B) Representative immunofluorescence of WT and ER-xL cortical neurons at DIV7, with active caspase-3 and tau.

(C) Quantification of neurite active caspase-3-positive neurons at DIV7. Data show average value \pm SEM.

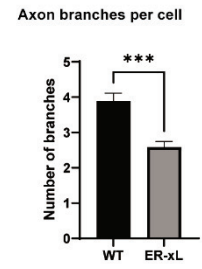
(D) Quantification of active caspase-3 positive surface per neuron at DIV7. Data show average value \pm SEM.

Statistical analysis: Student t-test. Data are from three independent experiments. (C) $N_{WT} = 37$, $N_{ER-xL} = 34$ (D) $N_{WT} = N_{ER-xL} = 24$.

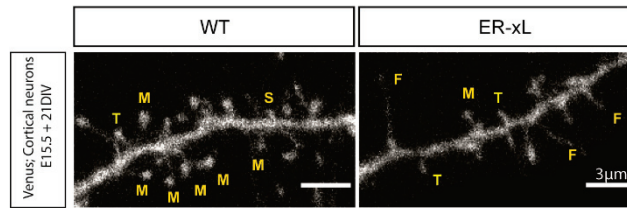
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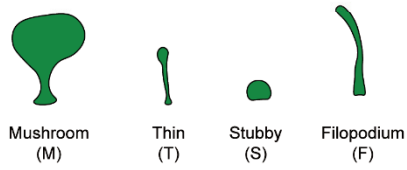
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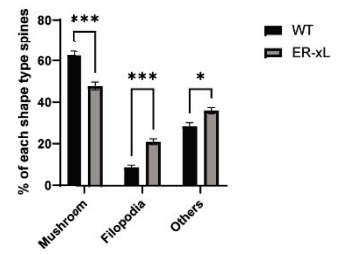
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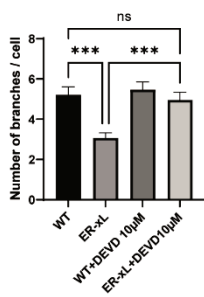
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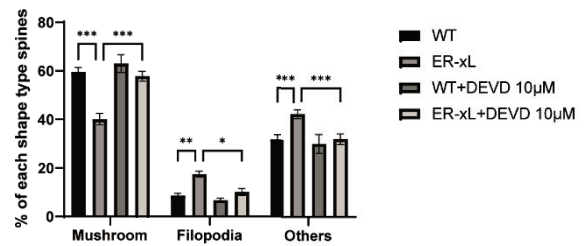


Figure 4. Mitochondrial Bcl-xL is required for axon branching and dendritic spine maturation

(A) Representative cortical neurons (inverted LUT) from dissociated cultures isolated from WT and ER-xL embryonic cortex after DIV5. *Ex vivo* cortical electroporation with a Venus-expressing plasmid followed by cortical neuron dissociation was performed at E15.5. Each axonal branch is indicated by an arrowhead.

(B) Quantification of number of axon branches per cell of DIV5 cortical neurons. Data show average value \pm SEM from three independent experiments. Mann-Whitney test was used for statistical analysis: $N_{WT} = 219$, $N_{ER-xL} = 250$.

(C) Representative images of dendritic spines of WT and ER-xL cortical neurons from dissociated cultures isolated from embryonic cortex after 21DIV. *Ex vivo* cortical electroporation with a Venus-expressing plasmid followed by cortical neuron dissociation was performed at E15.5. Representative spine morphology is indicated: M for mushroom, T for thin, S for stubby, and F for Filopodium.

(D) Schematic diagram for four spine morphologies: mushroom, thin, stubby, and filopodium.

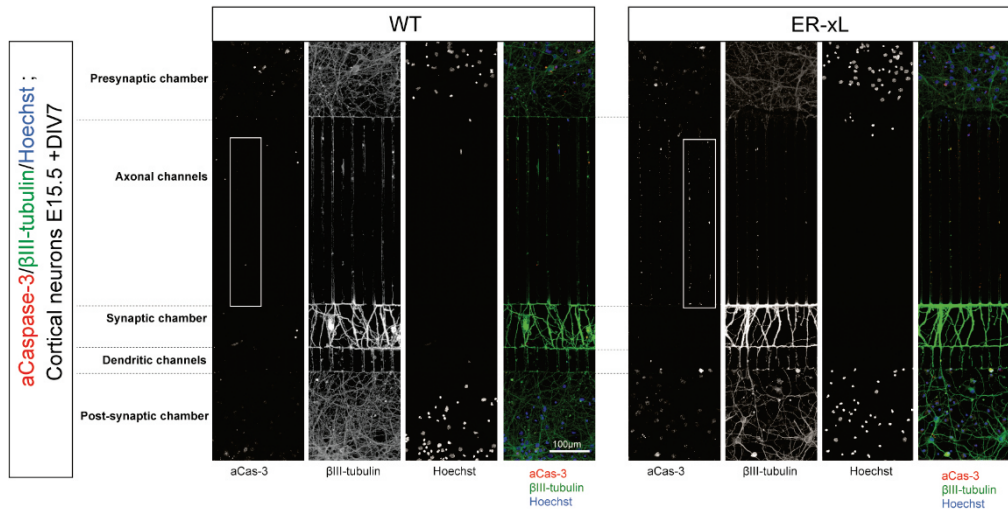
(E) Quantification of mushroom-shaped spine percentage, filopodial and other shape spine percentage of 21DIV cortical neurons. Data show average value \pm SEM from three independent experiments. Two-way ANOVA test was used for statistical analysis: $N_{WT} = 33$, $N_{ER-xL} = 65$.

(F) Histogram showing the effect of caspase-3 inhibitor Z-DEVD-FMK (10 μ M) on the number of axon branches per cell of cortical neurons at DIV5. The treatment with Z-DEVD-FMK was applied from DIV3-5. Data show average value \pm SEM from three independent experiments. Kruskal-Wallis test was used for statistical analysis: $N_{WT} = 143$, $N_{ER-xL} = 140$, $N_{WT+DEVD} = 122$, $N_{ER-xL+DEVD} = 122$.

(G) Histogram showing the effect of caspase-3 inhibitor Z-DEVD-FMK (10 μ M) on the spine shape percentage of cortical neurons at DIV21. The treatment with Z-DEVD-FMK was applied

from DIV14-21. Data show average value \pm SEM from three independent experiments. Two-way ANOVA test was used for statistical analysis: $N_{WT} = 22$; $N_{ER-xL} = 29$; $N_{WT+DEVD} = 13$; $N_{ER-xL+DEVD} = 23$.

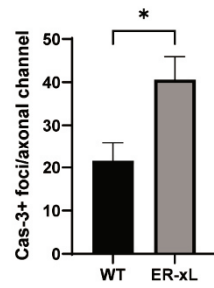
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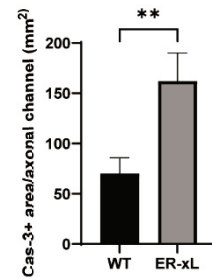
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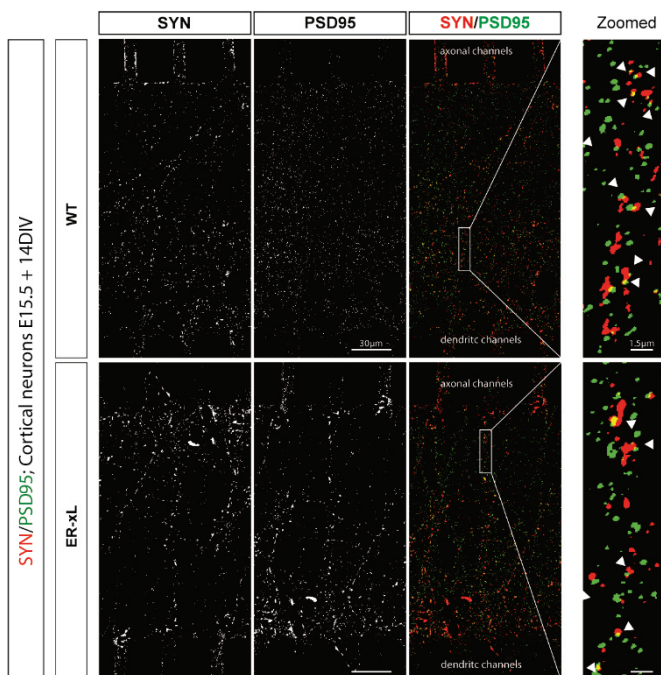
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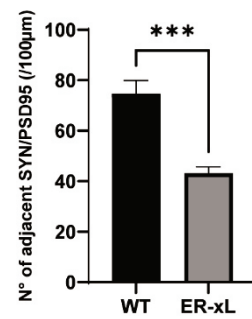


Figure 5. Mitochondrial Bcl-xL regulates synapse formation

(A) Representative immunofluorescence of WT and ER-xL cortical neurons in the 3-compartment microfluidic device with active caspase-3 and β III-tubulin. Nuclei were marked with Hoechst.

(B) Higher-magnification images of active caspase-3 signals in the axonal channel zone boxed in (A). Arrows point to active caspase-3-positive foci.

(C-D) Quantification of caspase-3-positive foci (E) or area (F) per axon channel at DIV7. Data are from at least three independent experiments. Average value \pm SEM is shown. Student t-test was used for statistical analysis: $N_{WT} = 79$, $N_{ER-xL} = 84$.

(E) Representative immunofluorescence of pre-synaptic protein SYN and post-synaptic protein PSD95 in the synaptic chamber between cortical neurons at 14DIV. Zoomed panels show high magnification images in the zone boxed in the SYN/PSD95 panels. Arrowheads point to adjacent spot SYN/PSD95.

(F) Quantification of adjacent spot SYN/PSD95 per 100 μ m of the axon of 14DIV cortical neurons. Data are from at least three independent experiments. Average value \pm SEM is shown. Student t-test was used for statistical analysis: $N_{WT}=N_{ER-xL} = 13$.

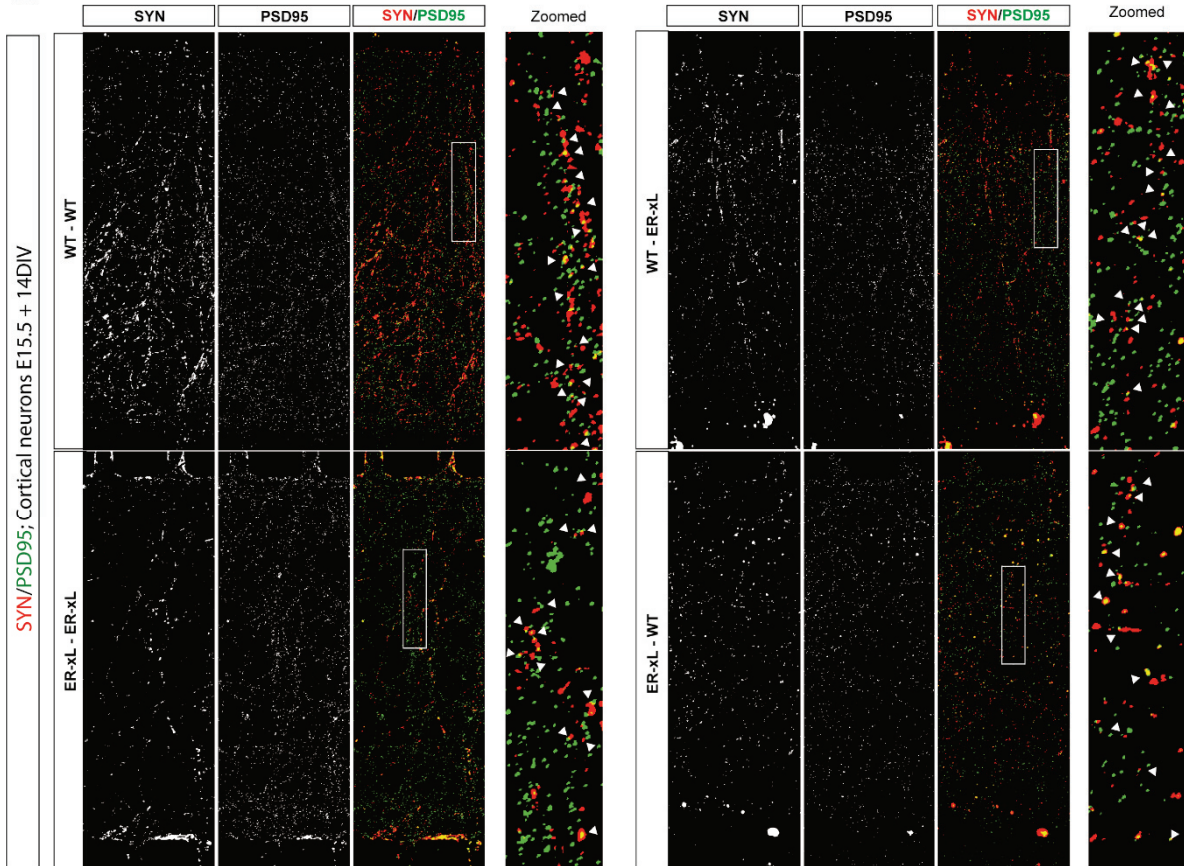
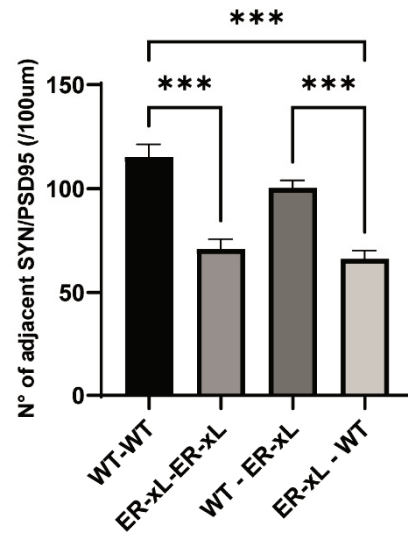
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Figure 6. Mitochondrial Bcl-xL regulates synaptogenesis at pre-synaptic sites

(A) Representative immunofluorescence of pre-synaptic protein SYN and post-synaptic protein PSD95 in the synaptic chamber between cortical neurons at 14DIV of WT – WT, ER-xL – ER-xL, WT – ER-xL, and ER-xL – WT. Zoomed panels show high magnification images in the zone boxed in the Syn/PSD95 panels. Arrowheads point to adjacent spot SYN/PSD95.

(B) Quantification of adjacent spot SYN/PSD95 per 100 μm of axon of 14DIV cortical neurons. Data are from three independent experiments. Average value \pm SEM is shown. Kruskal-Wallis test was used for statistical analyses: $N_{\text{WT-WT}}=54$, $N_{\text{ER-xL-ER-xL}}=50$, $N_{\text{WT-ER-xL}}=51$, $N_{\text{ER-xL-WT}}=49$.

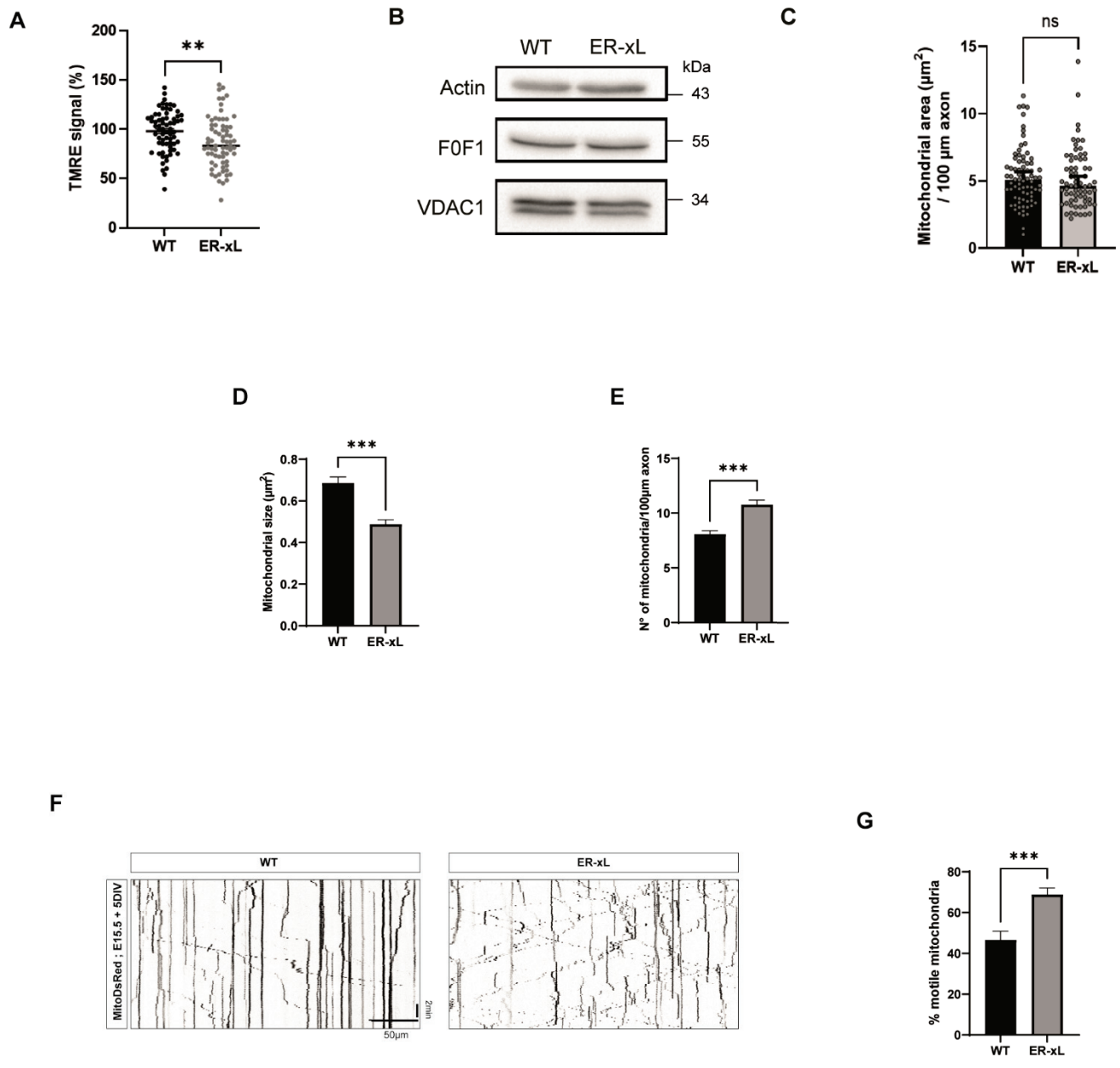


Figure 7. Loss of mitochondrial Bcl-xL induces changes in axonal mitochondrial morphology, dynamics, and potential

(A) Quantification of mitochondrial membrane potential by TMRE of axonal mitochondria in WT and ER-xL neurons at DIV5. Each point represents the average value for a given neuron (\pm SEM). Data are from three independent experiments. Student t-test was used for statistical analysis. $N_{WT} = 68$, $N_{ER-xL} = 71$.

(B) Expression of mitochondrial proteins in neurons at DIV5 derived from the cortex of WT and ER-xL E15.5 cerebral cortices. Western blot analysis was performed with indicated antibodies. Actin was used as the loading control.

(C-E) Quantification of area occupied by mitochondria per 100 μ m of the axon (C), mitochondria size (D), and number of mitochondria per 100 μ m axon (E) of the cortical neuron at DIV5. Neurons were electroporated at E15.5 by electroporation *ex vivo* with Venus to mark the axon and mitoDsRed to label mitochondria. Data show average values \pm SEM. Data are from three independent experiments. Mann-Whitney test was used for statistical analysis. $N_{WT} = 79$, $N_{ER-xL} = 69$.

(F) Representative kymographs of axonal mitochondria in dissociated cortical neurons at DIV5. Neurons were electroporated with MitoDsRed and mVenus.

(G) Quantification of motile axonal mitochondria. Data show average values \pm SEM. Data are from three independent experiments. Student t-test was used for statistical analysis. $N_{WT} = 19$, $N_{ER-xL} = 20$.

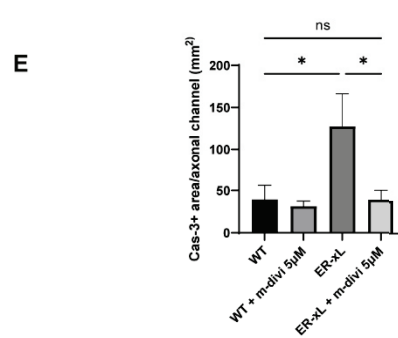
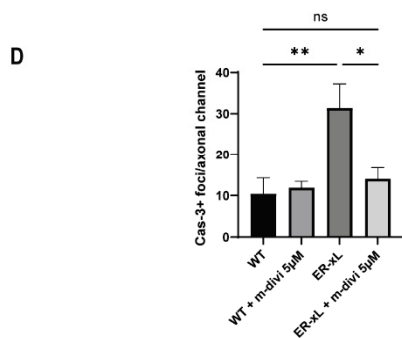
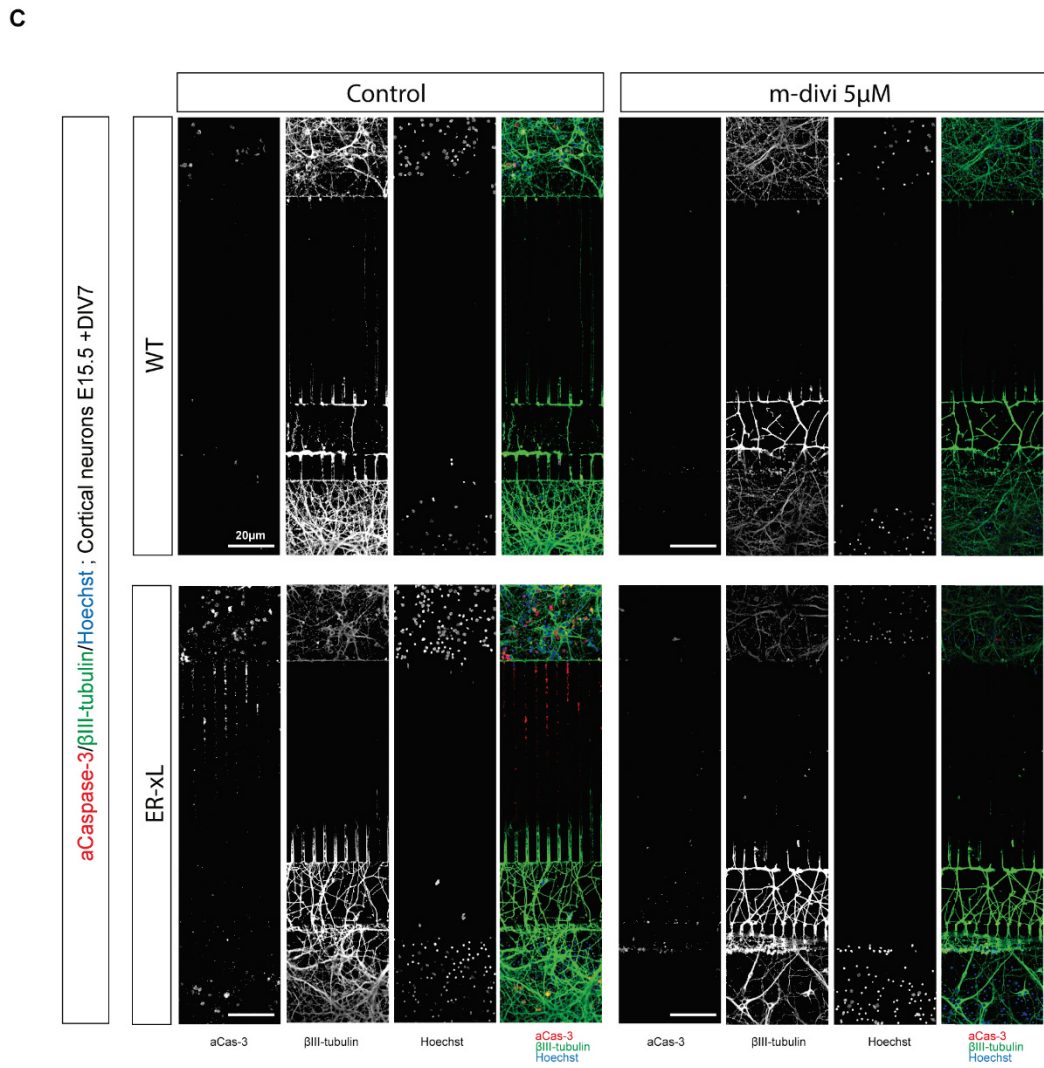
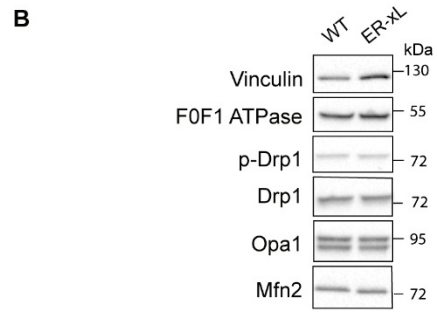
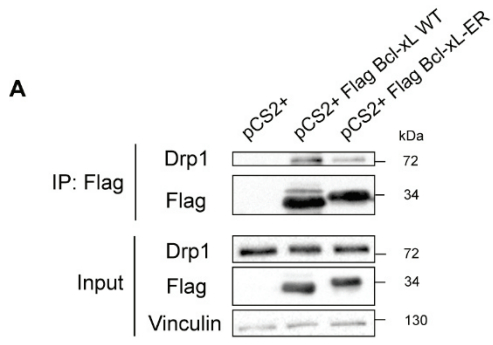


Figure 8. Mitochondrial Bcl-xL regulates axonal local caspase-3 activation via Drp-1 interaction

(A) Immunoblot analysis of co-immunoprecipitation between Flag-tagged Bcl-xL WT and Bcl-xL-ER with Drp-1 in HeLa cells.

(B) Expression of mitochondrial fusion and fission proteins in the cortical neurons at 7DIV. Western blot analysis was performed with indicated antibodies. Vinculin is used as the loading control.

(C) Representative images of cortical neurons in the microfluidic device at 7DIV: WT without treatment (control), WT with m-divi 5 μ M from 5DIV, ER-xL without treatment, (control) and ER-xL with m-divi 5 μ M from 5DIV.

(D-E) Quantification of caspase-3-positive foci (G) or area (H) per axon channel at DIV7. Data are from three independent experiments. Average value \pm SEM is shown. ANOVA test was used for Statistical analysis. $N_{WT}=N_{WT+m-divi} = N_{ER-xL+m-divi} = 7$, $N_{ER-xL} = 6$.

A

	WT	HET	ER-xL	Total	p-value
Observed	26 (28.9%)	44 (48.9%)	20 (22.2%)	90*	0.66**
<i>Expected</i>	22.5 (25%)	45 (50%)	22.5 (25%)		

* Newborns from 10 litters; ** Chi square test

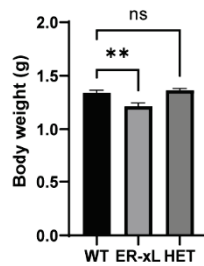
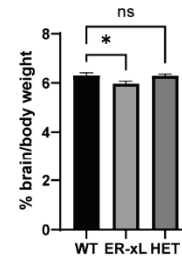
B**C**

Figure S1. ER-xL mice were born at Mendelian ratio with smaller size

(A) Table showing observed and expected number and percentage (in brackets) of WT, ER-xL, and HET newborns from heterozygous mating. Comparison between observed and expected distribution is performed by Chi square test, showing ER-xL newborn frequency follows Mendelian ratio.

(B) Body weight of WT, ER-xL, and heterozygous (HET) newborns at P1. Average value \pm SEM is shown. Kruskal-Wallis test was used for statistical analyses. Data are from 8 mouse litters. $N_{WT} = 17$, $N_{ER-xL} = 14$, $N_{HET} = 35$.

(C) Weight of WT, ER-xL, and HET newborn brains (normalized against individual body weight) at P1. Data are from 8 mouse litters. Average value \pm SEM is shown. Kruskal-Wallis test was used for statistical analysis. $N_{WT} = 17$, N_{ER} .

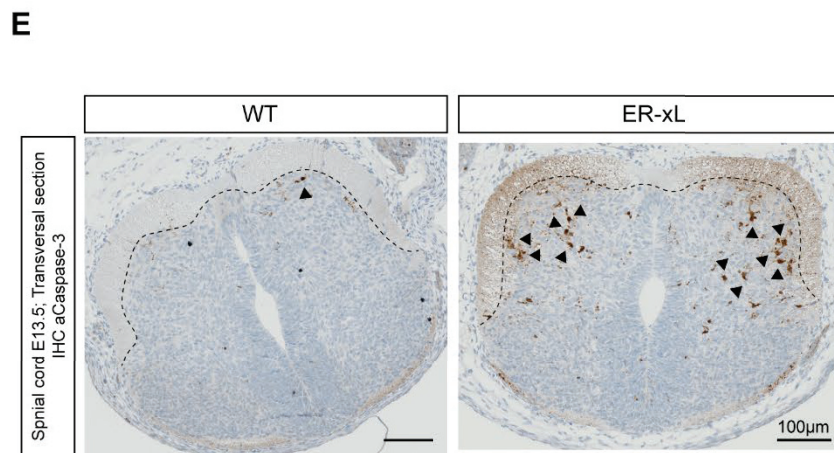
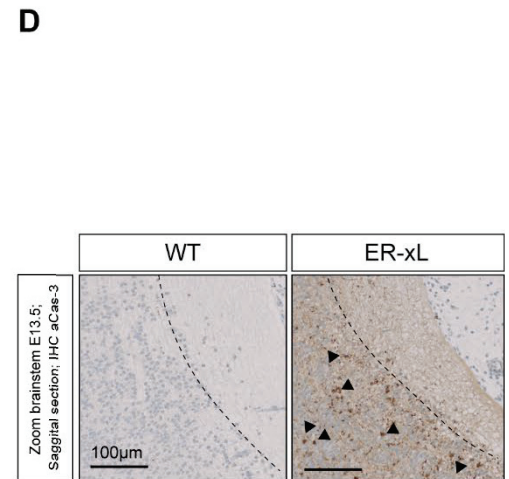
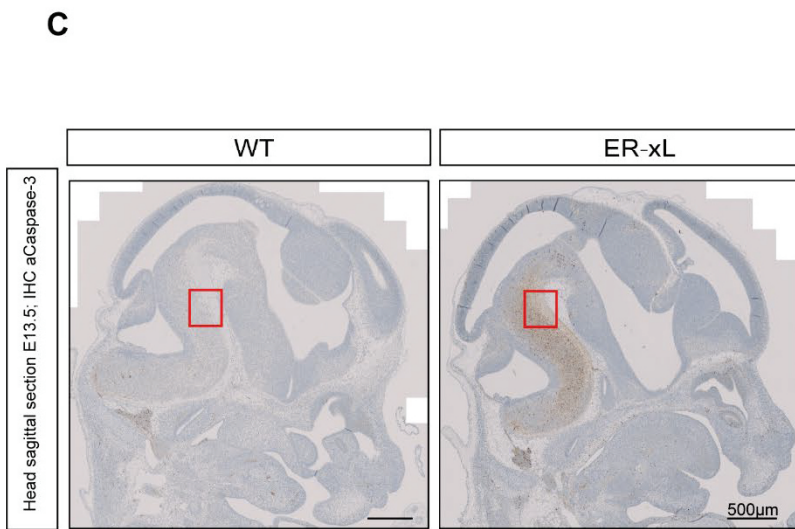
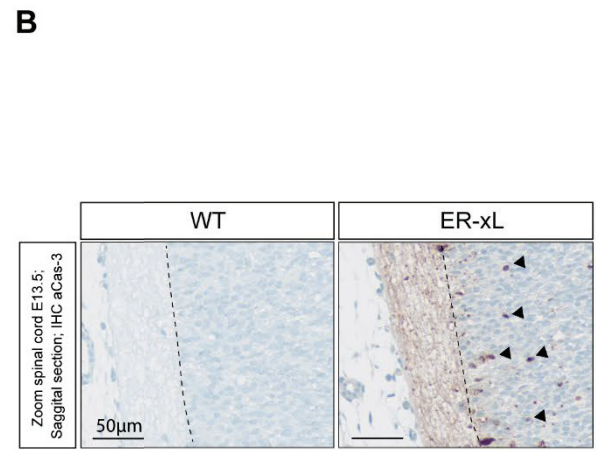
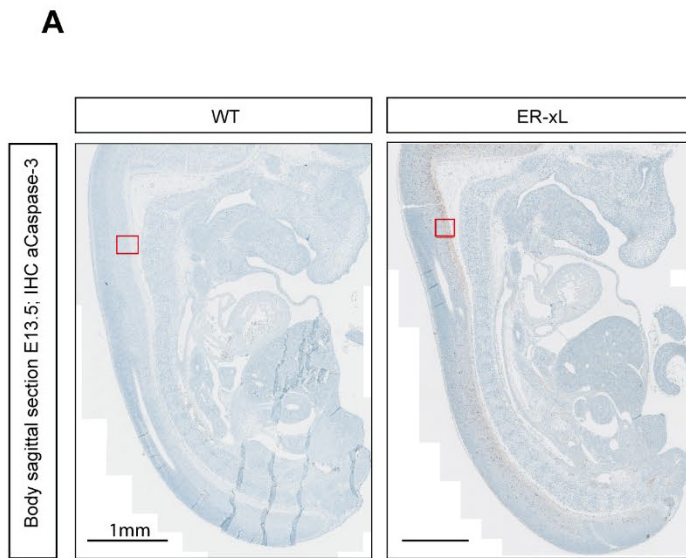


Figure S2. Immunohistochemistry analysis of E13.5 embryos

(A) Representative immunohistochemistry (IHC) of cleaved caspase-3 in the sagittal body section of WT and ER-xL E13.5 embryos.

(B) Higher-magnification images of the spinal cord region corresponding to the red box in A. Arrowheads point to active caspase-3-positive cell bodies.

(C) Representative IHC of cleaved caspase-3 in the sagittal head section of WT and ER-xL E13.5 embryos.

(D) Higher-magnification images of the brainstem region corresponding to the red box in B. Arrowheads point to active caspase-3-positive cell bodies.

(E) Representative of axial section of the spinal cord of WT and ER-xL embryos, with IHC of cleaved caspase-3. Arrowheads point to active caspase-3-positive cell bodies.

Data are representative of three independent embryo analysis for each genotype.

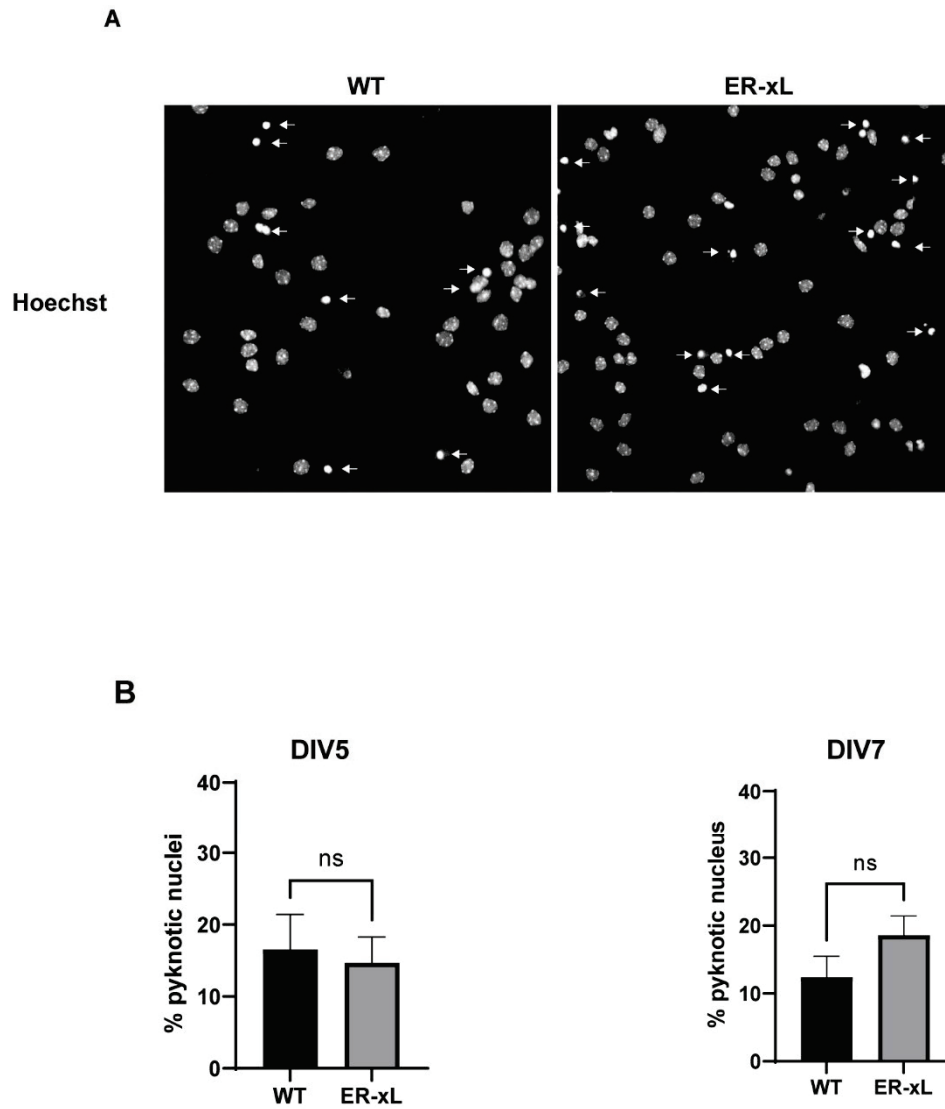


Figure S3. Mitochondrial Bcl-xL lack does not induce neuronal death

(A) Representative images of neuron culture nuclei marked by Hoechst at DIV5. Arrows point to representative pyknotic nuclei.

(B) Quantification of pyknotic nuclei of WT and ER-xL cortical neuron culture at DIV5 (Left panel) and DIV7 (Right panel). Data show average value \pm SEM. Student t-test was used for statistical analysis: $N_{WT} = 3$ $N_{ER-xL} = 3$.

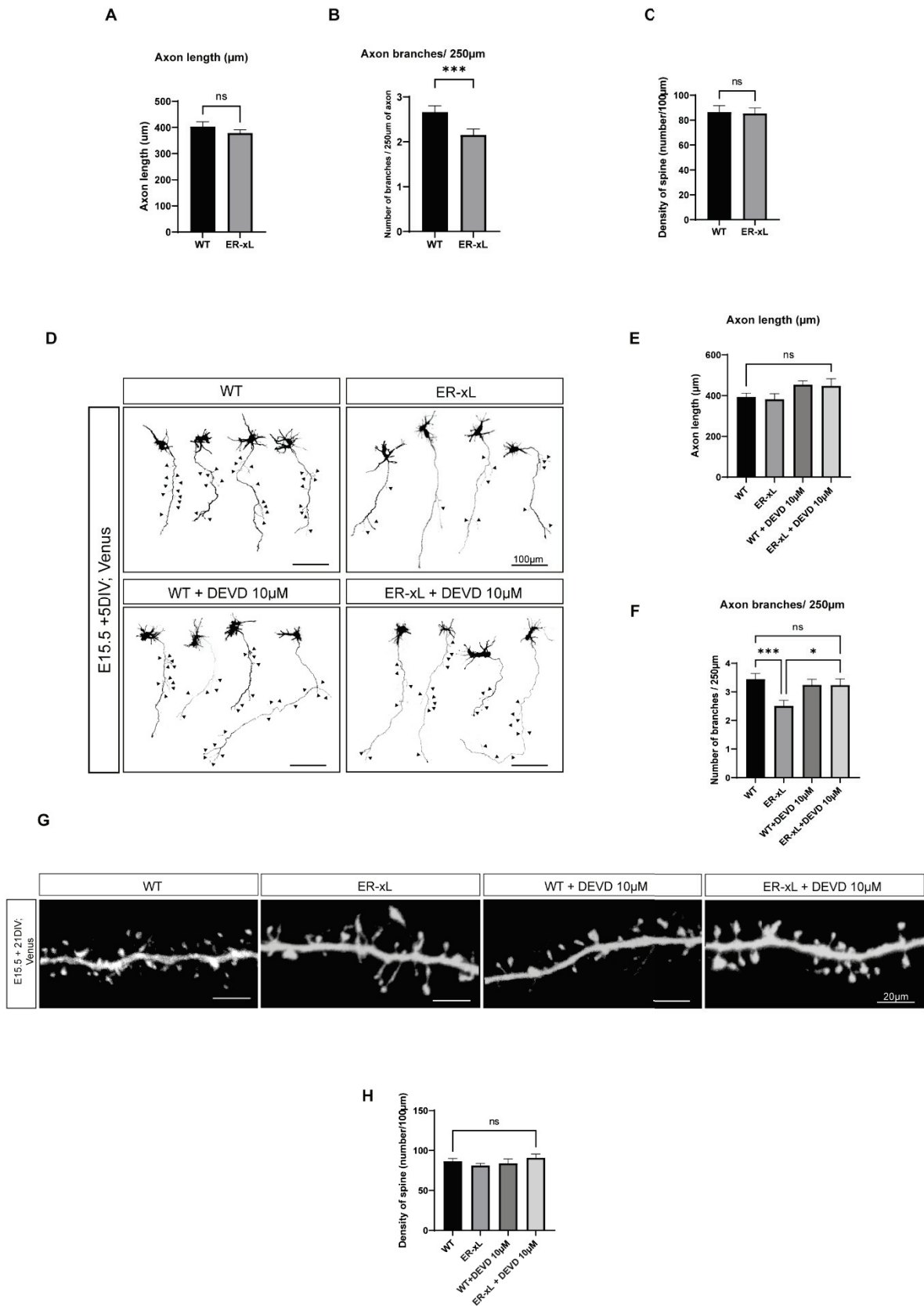


Figure S4. Mitochondrial Bcl-xL regulates axon branching and synaptogenesis

(A-B) Quantification of axon length (A) and number of axon branches per 250 μm of axon (B) of DIV5 cortical neurons. Data are from three independent experiments. Average value \pm SEM. is shown. Mann-Whitney test was used for statistical analysis: $N_{\text{WT}} = 219$, $N_{\text{ER-xL}} = 250$.

(C) Quantification of dendritic spine density of DIV21 cortical neurons. Data are from three independent experiments. Average value \pm SEM. is shown. Student t-test test was used for statistical analysis: $N_{\text{WT}} = 33$, $N_{\text{ER-xL}} = 51$.

(D) Representative images of 5DIV neurons from WT and ER-xL cortex without or with treatment with caspase-3 inhibitor Z-DEVD-FMK 10 μM from DIV3. Arrowhead indicates axon branch. Neurons were electroporated with Venus, followed by cortex dissociation at E15.5.

(E-F) Quantification of axon length (E) and number of axon branches per 250 μm of the axon (F) of cortical neurons at DIV5 without treatment or treated with Z-DEVD-FMK. Data are from three independent experiments. Average value \pm SEM. is shown. Kruskal-Wallis test was used for statistical analysis: $N_{\text{WT}} = 143$, $N_{\text{ER-xL}} = 140$, $N_{\text{WT+DEV D}} = 122$, $N_{\text{ER-xL+DEV D}} = 122$.

(G) Representative of images of dendrites with dendritic spines from DIV21 cortical neurons cultured from the isolated cortex of E15.5 WT and ER-xL embryos without treatment or ER-xL embryos with the treatment of caspase-3 inhibitor Z-DEVD-FMK 10 μM from DIV14. Neurons were electroporated with Venus to assess their morphology.

(H) Quantification of spine density of DIV21 neurons. Data are from three independent experiments. Average value \pm SEM is shown. One-way ANOVA test was used for statistical analysis: $N_{\text{WT}} = 22$; $N_{\text{ER-xL}} = 29$; $N_{\text{WT+DEV D}} = 13$; $N_{\text{ER-xL+DEV D}} = 23$.

EXPERIMENTAL PROCEDURES

Animals

Heterozygous ER-xL knock-in mice were generated on a C57BL/6J background. These mice express mouse Bcl-xL exon 3 with a modified transmembrane domain-containing an ER-targeting Cb5 sequence.

Animal care was in accordance with the recommendation of the European Community and the French National Committee. Animals had access to food and water *ad libitum* and were kept at a constant temperature on a 12-hour light/dark cycle.

***Ex vivo* electroporation**

DNA plasmid at 1 µg/µL mixed with 0.5% Fast Green (Sigma, 1:10 ratio) was injected into the lateral ventricles of isolated E15.5 embryonic mouse heads that were decapitated and reserved in complete Hank's Balanced Salt Solution (HBSS) (Life Technologies). Electroporation was performed on the whole head with skull and skin intact, using gold-coated electrodes (GenePads BTX) and the ECM 830 electroporator system (BTX). Electroporation parameters were: five unipolar pulses of 100 ms in length, with a 100 ms interval between each pulse. After electroporation, the brain was immediately used for culture preparation.

Primary cortical neuron culture

Cortices from E15.5 mouse embryos were dissected in sterile complete HBSS (cHBSS) supplemented with HEPES 2.5 mM (Sigma, 11560496), CaCl₂ 1 mM (Sigma), MgSO₄ 1 mM (Sigma), NaHCO₃ 4 mM (Sigma), and D-glucose 30 mM. After dissection, cortical parts were dissociated by incubating with papain 10 units/mL in cHBSS for 15 min at 37°C, followed by 1 min of incubation with DNase 1,500 units/mL in cHBSS. After incubation, cortices were washed three times with cHBSS and mechanically dissociated by repeatedly pipetting in cHBSS. After dissociation, cells were plated at 100,000 cells per 12 mm coverslip or 300,000

cells per 35 mm disk (Ibidi) coated with Poly-D-lysine (0.1 mg/mL, Sigma) in Neurobasal medium supplemented with B27 1x (Life Technologies), N2(1x), L-glutamine (0.5mM), 2-mercaptoethanol (50 μ M), and Penicillin/Streptomycin, referred to as complete NB, for 5-7 days. For long-term cultures of 21 days, cells were cultured in complete NB supplemented with fetal bovine serum (5%) for the first 7 days, then the serum-containing medium was gradually replaced by a serum-free medium from day 7 to day 14. From day 14 to 21, medium was changed each 3 days.

Microfluidic chamber preparation

Microfluidic chambers were prepared as previously described (Virlogeux et al., 2018). Briefly, microfluidic chambers were made of polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning). A mixture of 10:1 of liquid polymer and curing agent was poured into an epoxy resin mold. The filled molds were incubated under vacuum for 1 h to remove air bubbles, then incubated at 60°C for 3 h. Polymerized PDMS microchambers were pulled out, washed with alcohol 100% with quick passages through an ultrasonic bath, and finally washed with distilled water. Microchambers were dried at 60°C for 30 min. Dry microchambers and 35 mm glass-bottom disks (FluoroDish, WPI) were activated by a plasma cleaner treatment for 30 s. PDMS pieces were immediately fixed on a 35 mm disk. Microchambers were then sterilized under UV light for 30 min. The microchambers were coated with Poly-D-lysine (0.1 mg/mL, Sigma) in the upper and synaptic chamber, and a mixture of poly-D-lysine (0.1 mg/mL, Sigma) and laminin (10 μ g/mL) in the lower chamber overnight at 4°C. The next day, microchambers were washed three times with complete NB and placed at 37°C before plating.

Western blotting

Cells were washed in cold PBS (1X) buffer and then harvested in ice-cold lysis buffer containing 50 mM Tris pH 7.5, 150 mM sodium chloride, 1% NP40, 0.25% sodium deoxycholate, 1 mM EDTA, supplemented with protease inhibitor cocktail (Roche). Proteins

were separated by SDS-PAGE and then transferred to the PDVF membrane using Trans-blot® Turbo™ Transfer System. After transfer, the membrane was blocked in 5% milk in tris buffered saline (25 mM Tris-base pH 7.4, 137 mM sodium chloride, and 2.7 mM potassium chloride) plus 0.1% Tween®20 (TBS-T). After blocking, the membrane was incubated overnight at 4°C with the primary antibody in 5% milk in TBS-T. The next day, the membrane was washed three times with TBS-T and incubated with appropriate Horseradish peroxidase-conjugated secondary antibodies at the ratio of 1:3,000 in TBS-T at room temperature for 1 h. After washing three times with TBS-T, proteins were visualized with Lumi-Light^{PLUS} Western Blotting Substrate (Roche) using ChemiDoc Imaging System (Biorad). Primary antibodies used for Western blot were: Opa1 (1:1,000, BD Biosciences, 612607), Drp1 (1:500, Invitrogen, PA1-16987), Vinculin (1:500, Santa Cruz, sc-55465), VDAC1 (1:1,000, Abcam, ab14734), Actin (1:100, Sigma, A2066), phospho-Drp1 Ser616 (1:1,000, Cell Signaling, 4494), Mitofusin 2 (1:1,000, Cell Signaling, 9482), Bcl-xL (1:1,000, Cell Signaling, 2764), Calnexin (1:1,000, Abcam, ab22595), F0F1 ATPase (1:2000, BD Biosciences, 612518), Flag M2 (1:1,000, Sigma, F3165).

Co-immunoprecipitation

Immunoprecipitation experiments were performed on HeLa cells. Cells at 89-90% confluence in 6-well plates were transfected with 2 µg of pCS2+ empty vector, pCS2+Flag-Bcl-xL WT or pCS2+Flag-Bcl-xL-cb5, using X-tremeGENE™ HP DNA Transfection Reagent (Roche) according to the manufacturer's protocol. After 24 h of transfection, cells were harvested in the TNE buffer (10 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA (pH 7,4), supplemented with 0.2% NP-40, 2.5% sodium pyrophosphate, 0.1% glycerol-2-phosphate, and protease inhibitor). Subsequently, protein extracts were incubated with 20 µL of Anti Flag M2 magnetic beads (Sigma Aldrich) overnight at 4°C. Magnetic beads were then washed three times with TNE buffer and analyzed by immunoblot.

Subcellular fractionation

Subcellular fractionation experiments followed the previously described protocol (Wieckowski et al., 2009). Briefly, 8-10 brains isolated from newborn mice were washed twice in the PBS and cut into small pieces in ice-cold MB buffer (210 mM mannitol, 70 mM sucrose, 1mM EDTA, and 10mM Hepes (pH 7.4) containing protease inhibitors; Roche). Cells were homogenized and then disrupted using Dounce homogenizers (loose and tight) 20 to 30 times. The homogenized mixture was then centrifuged at 600 g to remove nuclei and undisrupted cells and centrifuged at 10,000 g to obtain crude mitochondria. The pellet containing crude mitochondria was suspended in MB buffer and then gently laid on a Percoll medium (225 mM mannitol, 25 mM HEPES (pH 7.4), 1 mM EGTA, and 30% Percoll (vol/vol)) and centrifuged for 30 min at 95,000 g separating the mitochondria-associated membranes (MAMs) from the mitochondria. The supernatant was centrifuged for 1 h at 100,000 g. After centrifugation, the pellet contained the microsome fraction, and the supernatant contained the cytosol fraction. Fifteen micrograms of each subcellular fraction and 50 µg of the total fraction were analyzed by Western blot.

TMRE labeling

For measurements of mitochondrial potential, neurons were pre-incubated in complete HBSS for 30 min at 37°C, then incubated in 20 nM tetramethylrhodamine ethyl ester (TMRE) in complete HBSS for 30 min at 37°C. The medium was then replaced by a 5 nM TMRE solution immediately before live imaging.

Immunofluorescence

Cortical neurons were seeded onto coverslips coated with Poly-D-lysine and laminin (Corning®). Neurons were maintained in culture for 5-21 days, depending on experiments (indicated in each experiment). Cells were then fixed in 4% paraformaldehyde solution for 20

min and washed three times with PBS. Following permeabilization with 3% bovine serum albumin in PBS 0.1% Triton X-100, cells were incubated at room temperature for 1 h with the primary antibodies. Goat anti-mouse or goat anti-rabbit immunoglobulin G Alexa Fluor 488 or 568 were used as secondary antibodies (Molecular Probes) at 1:1,000 dilution for 1 h. Nuclei were visualized using Hoechst 33342 dye (Invitrogen H3570) at 1:10,000 dilution.

Immunofluorescence of microfluidic chambers was performed following the protocol for neuron culture. Incubation with the primary antibodies was performed at 4°C overnight.

For preparing cryosections, brains were isolated from newborn mice and fixed in 4% paraformaldehyde solution. After fixation, brain samples were washed three times with PBS and then cryo-protected with a range of 10%, 20%, and 30% sucrose solutions. Brains were frozen rapidly in OCT medium (TBS), cut into 10 µm-thick slices, and preserved at -20°C. Following 10 min of thawing, brain slices were blocked with Antibody Diluent (ThermoFisher, 003118). Slides were incubated at 4°C overnight. Goat anti-mouse or goat anti-rabbit immunoglobulin G Alexa Fluor 488 or 568 were used as secondary antibodies. Slides were then mounted with VECTASHIELD® with DAPI (Vectalabs, H-1200).

The primary antibodies used: cleaved caspase-3 (1:400, Cell Signaling, 9661), βIII-tubulin (1:1,000, Biolegend, 801201), tau (1:200, Abcam, ab80579), Post Synaptic Density Protein 95 (1:1,000, Chemicon®, MAB1598), Synaptophysin (1:200, Abcam, ab14692).

Immunohistochemistry

Mouse embryos were collected from euthanized pregnant mice and fixed in 4% paraformaldehyde solution. After fixation, embryos are rinsed 3 times with PBS and then embedded in a paraffin block. Embryo paraffin blocks were then sectioned at 4µm thickness and transferred onto glass slides. For immunohistochemistry, embryo sections were deparaffinized by xylene and re-hydrated through 100%, 95%, 70%, and 50% alcohols. The epitope retrieval was realized using a Tris-EDTA buffer pH7.8 during 36 min. Slide sections

were then incubated with the primary antibody cleaved caspase-3 (1:400, Cell Signaling, 9664) at 37°C for 1h. Goat anti rabbit was used as secondary antibody (1:300, Vector BA1000). Signals were revealed by using the DABMap kit (Roche, 05266360001). Immunohistochemistry steps were performed in the Roche Discovery XT system.

Image acquisition and analysis

Confocal images were acquired on a Zeiss LSM 880 system equipped with Airyscan, with these objective lenses: 10x AN 0.45, 20x AN 0.8, and 63x AN 1.4. For the axonal morphogenesis analysis, we performed large-field acquisition of a 4×4 tiling scan using a 20x objective. All neurons on the reconstituted image were quantified, and axon length was measured using ImageJ.

During live imaging, the temperature was maintained at 37°C and CO₂ at 5% v/v.

Statistics

Statistical analyses were performed using Prism (GraphPad). Statistical tests and number of replicates are indicated in the figure legends.

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DISCUSSION & PERSPECTIVES

Role of Bcl-xL mitochondrial pool in mouse embryonic development

The key role of BclxL in development was demonstrated by Motoyama and colleagues, who showed that the invalidation of *bclx* is embryonic lethal (Motoyama et al., 1995). Using the present ER-xL knock-in mouse model, we established that the maintenance of the ER pool of Bcl-xL, while Mitochondria-addressed Bcl-xL is absent, apparently restores normal embryonic development since ER-xL mice come to birth without any visible morphological abnormalities. Furthermore, ER-xL newborns survive up to 12 hours and show normal mobility, suggesting that they do not suffer from severe impairment of respiratory and locomotor systems. In addition, morphological and histological analyses at E13.5 and E17.5 stages did not reveal any anomalies in major organs, namely the heart, the liver, the kidneys, the spleen, and the lungs. However, regarding the brain and the spinal cord, significant caspase-3 activation was observed in the white matter but not in the grey matter. Of note, primary cultures of ER-xL cortical neurons did not reveal a higher rate of cell death, suggesting that the observed caspase-3 activation was independent of apoptosis. In fact, this observation also suggests that the massive apoptosis observed in *bclx*-null mice by Motoyama and colleagues might be repressed or delayed in ER-xL mice. Another possibility would be that apoptosis in ER-xL mice increases but to lower levels compared to *bclx* KO mice. It would be worth testing this hypothesis by precisely comparing the apoptosis pattern of both mouse models and monitoring apoptotic features downstream of caspase activation, such as pyknotic nuclei, TUNEL staining, and detection of karyorrhectic debris and distended phagocytic cells (see, Motoyama et al., 1995).

Contribution of ER Bcl-xL in mouse embryonic development

Why loss of mitochondrial Bcl-xL does not induce embryonic lethality in ER-xL mice remains an open question. Our observations suggest that the ER pool of Bcl-xL prevents embryonic lethality in the absence of mitochondrial Bcl-xL. In this respect, the exact mechanisms by which ER Bcl-xL delays this fatal phenotype remain to be investigated.

Even though mitochondria are considered the main action site of Bcl-xL regarding its anti-apoptotic activity, non-mitochondrial Bcl-xL has been demonstrated to contribute to its overall protective effect concerning cell death (Eno et al., 2012). In fact, our observations suggest that ER-Bcl-xL could have a protective role against stress induction that may occur during development. The underlying mechanisms that remain to be characterized may involve ER Ca^{2+}

trafficking and ER-stress regulatory pathway. Indeed, in the past few years, ER stress and the Unfolded protein response (UPR) pathway have been reported to contribute to neurogenesis, especially during corticogenesis (Cho et al., 2009; Laguesse et al., 2015). Perturbation in UPR signaling and ER protein quality control leads to aberrant cerebral cortex development (Mimura et al., 2008; Laguesse et al., 2015). Furthermore, UPR-regulated genes are expressed during early development (Murao and Nishitoh, 2017). For instance, ATF4 expression is high in the E10-12 mouse brain, drastically decreasing later on (Frank et al., 2010). Of note, the peak of UPR takes place before embryonic stage E13.5, when *bclx* KO embryos actually die (Motoyama et al., 1995; Frank et al., 2010).

In this respect, we actually found that mouse embryonic fibroblasts derived from ER-xL mice are more resistant to ER stress inducers. In contrast, Mito-xL fibroblasts (expressing Bcl-xL at the mitochondria but not the ER) were found to be more sensitive, confirming the protective effect of ER-Bcl-xL with respect to ER stress (Jabbour et al., BioRxiv).

Overall, these data support the idea that ER-addressed Bcl-xL is conceivably sufficient to regulate developmental UPR and block ER-stress-related cell death in the embryo.

Axonal caspase-3 activation

Strikingly, by immunohistochemistry (E13.5 spinal cord) and immunofluorescence (P1 brain), we showed that active caspase-3 levels are higher in the white matter than in the grey matter in both the spinal cord and the cerebral cortex of ER-xL embryos.

We thus hypothesized that this could reveal local caspase-3 activation in axons, but not the soma, of ER-xL neurons. Indeed, the culture of cortical neurons isolated from the cortex of ER-xL embryos (E15.5) showed no change in apoptotic body percentage after 5 days of culture *in vitro*. Moreover, we successfully cultivated these neurons for up to 21 days with proper differentiation and synapse formation. These observations confirmed that these neurons did not undergo apoptosis, although active caspase-3 was detected in the neurites, predominantly in axons, as shown using a microfluidic system. Collectively our results show that ER-xL neurons are prone to local caspase-3 activation in axons, independent of cell death.

It should be noted that we could detect local caspase-3 activation in WT neurons, although at lower levels. It is thus conceivable that caspase-3 activation naturally takes place in WT neurons, being amplified and expended in the absence of mitochondrial Bcl-xL and causing neural developmental defects. In fact, local caspase-3 activation has been described in the

developing CNS (Campbell and Holt, 2003; Cusack et al., 2013; Guo et al., 2016). In these models, caspase-3 plays different physiological roles in axon branching and axon and spine pruning. Caspase-3 deletion results in fewer axon branches and impacts axon and spine elimination *in vitro* and *in vivo*.

It would thus be interesting to assess caspase-3 activity using more sensitive biosensors based on fluorescence resonance energy transfer (SCAT3) (Takemoto et al., 2003). Live imaging carried out using this type of sensor will also allow following the correlation between axonal caspase-3 activation and neuron development and compare caspase-3 activity between WT and ER-xL neurons.

Axon branching and synaptogenesis

We observed *in vitro* that active caspase-3 signals are located preferentially in the branching point and contact sites between neurons. Thus, we hypothesized that aberrant local caspase-3 activation might result in abnormal axon development and synapse formation.

Indeed, morphological analysis of 5DIV ER-xL cortical neurons shows axon branch decrease without impacting axon length. The causality between caspase-3 activation and defect axon branching was demonstrated using the caspase-3 inhibitor, z-DEVD-fmk (which also inhibits caspase-7). Treatment of ER-xL neurons with z-DEVD-fmk restores the normal phenotype of axon branching, confirming that axon branch decrease is the consequence of local caspase-3 activation.

Paradoxically, a slight and transient local caspase-3 activation was reported to be essential for axon branch formation by Campbell and Okamoto (Campbell and Okamoto, 2013). Of note, other studies highlighted the role of caspase-3 in axon pruning, in which active caspase-3 removes unnecessary or improper connected axon arborization (Nikolaev et al., 2009; Cusack et al., 2013). Collectively these data suggest that, while baseline caspase-3 activity is critical for the design of axon arborescence, the levels of caspase-3 activity found in ER-xL neurons are, in contrast, presumably out of range resulting in deleterious effects on axonal development.

ER-xL neurons actually exhibit abnormalities in synapse formation and maturation. Synapse formation was analyzed by using the microfluidic device that permits the formation of proper connections between the axon and dendrites (Virlogeux et al., 2018). These microfluidic analyses suggested that the observed synaptic alterations depended on the presynaptic compartment, which is in accordance with our previous observation showing that the caspase-

3 signals are located principally in the axonal compartment of ER-xL neurons. In this respect, it should be reminded that Bcl-xL was reported to control vesicle trafficking in axon terminals; such control might thus be altered in an ER-xL context (Li et al., 2013).

The confinement between pre- and postsynaptic chambers allows us to determine which structure originates the connection disturbance (Virlogeux et al., 2018). We found an ER-xL-WT cortico-cortical network is sufficient to recapitulate the lack of synapse formation observed in the ER-xL-ER-xL network. In contrast, the WT-ER-xL network exhibits a synapse density similar to WT-WT one, suggesting synaptic alteration depends on the presynaptic compartment. This result is in accordance with our previous observation that the caspase-3 signals are located principally in the axon compartment of mutant neurons.

We further investigated synapse maturation by assessing the morphology of dendritic spines, a structure on the dendrites harboring the postsynaptic part of the glutamatergic synapse. ER-xL neurons showed a decrease in the number of mushroom-like spines, which are considered markers of the mature synapse. Treatment with z-DEVD-FMK restores the proportion of mushroom-like form, confirming that this alteration is due to local activation of caspase-3. Of note, filopodial spines, described as the precursors of dendritic spines, are increased in ER-xL neurons.

Mitochondrial morphology and $\Delta\Psi_m$

Bcl-xL, like other Bcl-2 anti-apoptotic proteins, is a safeguard of the mitochondria, protecting their integrity and optimizing their functions. Indeed, we observed important changes in mitochondrial dynamics in axons of ER-xL neurons.

Axonal mitochondria are fragmented and have a lower membrane potential ($\Delta\Psi_m$). According to the literature, mitochondrial fragmentation and $\Delta\Psi_m$ change are intimately correlated. In effect, a drop of $\Delta\Psi_m$ causes rapid mitochondrial dynamics modifications favoring their fusion (Ishihara et al., 2003; Miyazono et al., 2018; Umbaugh et al., 2021). In addition, $\Delta\Psi_m$ decrease inhibits mitofusin-mediated fusion (Legros et al., 2002), whereas mitochondria fission induces a reduction of $\Delta\Psi_m$ (Suzuki et al., 2018).

$\Delta\Psi_m$ decrease may impact on mitochondrial ATP production, which is essential for neural differentiation and development (Dimroth et al., 2000; Tao et al., 2014). In addition, Bcl-xL has been found to directly interact with F0F1 ATPase, optimizing ATP production (Alavian et

al., 2011). Thus, it is highly possible that mitochondrial ATP synthesis capacity is declined in ER-xL neurons.

However, these mitochondrial changes are presumably downstream of caspase-3 activation since ER-xL neurons treated with z-DEVD-fmk closely resemble WT ones, indicating that caspase activation is the upstream causing event with respect to the developmental defects observed in the ER-xL model system.

An interesting question would be why the axonal population of mitochondria is the most impacted by the lack of Bcl-xL. It is well-known that mitochondria adopt a tiny individual shape for shifting throughout the length of neural projections. Mitochondria are present as punctated round forms in the axon but have tubular structures in the dendrite (Popov et al., 2005; Lewis et al., 2018). The balance between fusion and fission in the axon is kept under control to maintain axonal mitochondrial morphology (Lewis et al., 2018; Di Meo et al., 2021). Such a high fission rate would be a favoring factor for generating inter-mitochondrial Bcl-2 protein heterogeneity and, thus, iMOMP. To test this hypothesis, fusion and fission rates in the axon should be measured in the control and ER-xL neurons. In addition, iMOMP status should be assessed in these neurons with a couple of fluorescent probes, GFP-FKBP and mitoCherry-FRB (Ichim et al., 2015).

Role of Drp1

The loss of Bcl-xL at the mitochondria induces mitochondria fragmentation, suggesting that Bcl-xL might negatively regulate Drp1-mediated fission. We thus took advantage of fluidic isolation of the microchamber to solely treat the axon with Drp-1 inhibitor, m-divi. ER-xL cortical network treated with m-divi displays fewer active caspase-3 signals, suggesting that mitochondrial fragmentation causes caspase-3 activation as previously observed in other cell systems (Tait and Green, 2008; Ichim et al., 2015; Kalkavan and Green, 2018). In these cases, sublethal caspase-3 signals are the result of iMOMP. Thus, Bcl-xL might also control local caspase-3 activation through iMOMP regulation in our model. Mitochondrial dynamics was recently featured as a potential mechanism to regulate the incomplete MOMP (iMOMP). By genetically engineering Mitofusin-1 and -2, the proteins in charge of mitochondrial fusion, Cao and coworkers propose that a fragmented mitochondrial network harbors more heterogeneity of Bcl-2 anti-apoptotic proteins. Consequently, the mitochondria with more anti-apoptotic proteins are protected against Bax accumulation and Bax/Bak-induced permeabilization (Cao et al., 2022).

Our data indicate that Bcl-xL, and presumably other Bcl-2 homologs, can control iMOMP *via* regulating the mitochondrial network. Although regulation of mitochondrial fusion and fission by Bcl-2 proteins is not a novel concept (Berman et al., 2009; Bonneaud et al., BioRiv 2021), we show here for the first time that Bcl-xL-regulating mitochondrial network controls sublethal caspase-3 activation.

Our results support the notion that the Bcl-xL activity on mitochondrial dynamics is mediated *via* Drp1 interaction. Indeed, co-IP experiments in HeLa cells demonstrated that ER-addressed Bcl-xL interacts less with Drp1 compared to Mito- addressed Bcl-xL. The interaction between Bcl-xL and Drp-1 possibly occurs at the MOM level but not in the cytosol. Indeed, even having lost its capacity to retrotranslocate to the cytosol, the Mito- addressed Bcl-xL protein, in which the TM domain is replaced by an ActA sequence, exhibits similar interaction with Drp1 compared to WT protein.

Mitochondrial Bcl-xL absence does not entirely abolish its interaction with Drp1, suggesting that the two proteins may interact outside of mitochondrial. Drp1-Bcl-xL interaction possibly occurs at the ER. Indeed, Drp1 is also recruited to the ER and assembles into oligomers. The Drp1 oligomers are consequently transferred to the mitochondrial and perform their function (Ji et al., 2017). However, this interaction between Bcl-xL and Drp1 at ER is likely not sufficient to suppress the activity of Drp-1 at the mitochondria.

Is calcium a player in the game?

Calcium trafficking has an impact on numerous pathways, including cell differentiation and cell death. In fact, Ca^{2+} and $\Delta\Psi_m$ share a complex relationship. On the one hand, mitochondrial Ca^{2+} uptake defines the $\Delta\Psi_m$ value. Moderate Ca^{2+} uptake boosts mitochondrial respiration and increase $\Delta\Psi_m$ (Robb-Gaspers et al., 1998). In contrast, overload of mitochondrial Ca^{2+} induces mitochondrial depolarization (Vergun et al., 1999). On the other hand, mitochondrial Ca^{2+} uptake *via* MCU is $\Delta\Psi_m$ -dependent (Williams et al., 2013).

Bcl-2 proteins, including Bcl-xL, were reported to regulate calcium homeostasis. Bcl-xL has been found to engage both ER and mitochondrial calcium. Notably, Bcl-xL interacts with IP3R at the ER and VDAC at the mitochondria. These two Ca^{2+} channels, directly linked by GRP78, form a straight Ca^{2+} transfer platform between the two organelles (Szabadkai et al., 2006). When Bcl-xL is absent from one side, as it is in our model, Ca^{2+} perturbations may occur. In this respect, we observed a decrease in $\Delta\Psi_m$ of the mitochondria of ER-xL axons. Thus, it would be interesting to compare the axonal mitochondrial Ca^{2+} levels of ER-xL and WT

neurons. These experiments should be linked to electron microscopy observations to measure the distance between the ER and mitochondria at the level of the axon (Qi et al., 2015; Giamogante et al., 2020).

In summary, our work demonstrated a novel non-apoptotic role of the protein Bcl-xL in the developing neuron. By confining the local caspase-3 activation, Bcl-xL regulates neural polarization and synapse formation. This functionality of Bcl-xL is related to Drp-1 mitochondrial network regulation. Our findings shed new light on the regulatory role of Bcl-2 proteins in the sublethal caspase-3 activation. That opens novel perspectives for the studies on Bcl-2 roles in the physiological and pathological contexts.

ANNEX I

EVOLUTIONARY BIOLOGY

Ancient and conserved functional interplay between Bcl-2 family proteins in the mitochondrial pathway of apoptosis

Nikolay Popgeorgiev^{1*†}, Jaisou D Sa^{2*}, Lea Jabbour¹, Suresh Banjara², Trang Thi Minh Nguyen¹, Aida Akhavan-E-Sabet², Rudy Gadet¹, Nikola Ralchev¹, Stéphen Manon³, Mark G. Hinds⁴, Hans-Jürgen Osigus⁵, Bernd Schierwater^{5,6}, Patrick O. Humbert², Ruth Rimokh¹, Germain Gillet^{1†}, Marc Kvansakul^{2†}

In metazoans, Bcl-2 family proteins are major regulators of mitochondrially mediated apoptosis; however, their evolution remains poorly understood. Here, we describe the molecular characterization of the four members of the Bcl-2 family in the most primitive metazoan, *Trichoplax adhaerens*. All four trBcl-2 homologs are multimotif Bcl-2 group, with trBcl-2L1 and trBcl-2L2 being highly divergent antiapoptotic Bcl-2 members, whereas trBcl-2L3 and trBcl-2L4 are homologs of proapoptotic Bax and Bak, respectively. trBax expression permeabilizes the mitochondrial outer membrane, while trBak operates as a BH3-only sensitizer repressing antiapoptotic activities of trBcl-2L1 and trBcl-2L2. The crystal structure of a trBcl-2L2:trBak BH3 complex reveals that trBcl-2L2 uses the canonical Bcl-2 ligand binding groove to sequester trBak BH3, indicating that the structural basis for apoptosis control is conserved from *T. adhaerens* to mammals. Finally, we demonstrate that both trBax and trBak BH3 peptides bind selectively to human Bcl-2 homologs to sensitize cancer cells to chemotherapy treatment.

INTRODUCTION

Apoptosis represents one of the hallmarks of multicellularity in the animal kingdom (1). This process shapes the body during embryonic development, maintains tissue homeostasis, and eliminates undesirable or potentially harmful cells from the organism (2). Proteins belonging to the Bcl-2 family are critical arbiters of apoptosis. Found originally in chromosomal translocations in follicular lymphomas, Bcl-2 homologs have now been found in almost all metazoans (3). Genetic screening of cell death mutants performed initially in nematodes and subsequent studies in cnidarians, arthropods, platyhelminthes, and vertebrates uncovered a number of Bcl-2-related proteins (4–8). These proteins are characterized by the presence of one to four conserved Bcl-2 (BH) motifs, as well as a hydrophobic C-terminal transmembrane (TM) motif, allowing them to be localized to intracellular membranes (9). Bcl-2 proteins are divided into members that either promote or oppose apoptosis. Proapoptotic Bcl-2 proteins are further subdivided into multimotif Bcl-2 homologs and the phylogenetically separate BH3-only proteins, which have only the BH3 motif (10).

It is generally accepted that Bcl-2 family proteins were evolutionarily selected to control cell survival in metazoans (11). However, the control of apoptosis differs substantially between roundworms and

vertebrates. Seminal experiments conducted in *Caenorhabditis elegans* demonstrated that the Bcl-2 protein Ced-9 binds to the caspase-activating Apaf-1 homolog Ced-4. Apoptosis ensues when the BH3-only protein Egl-1 is up-regulated to competitively bind to Ced-9, thus releasing Ced-4 and leading to the activation of the caspase Ced-3. In vertebrates, however, Bcl-2 proteins are found to control a key step in the induction of apoptosis called mitochondrial outer membrane permeabilization (MOMP) (12). Antiapoptotic Bcl-2 homologs including Bcl-2, Bcl-w, Bcl-x_L, Mcl-1, and A1/Bfl-1 repress proapoptotic Bax and Bak, which the effect release of cytochrome c and subsequent caspase activation through their oligomerization and perforation of the MOM (13). Proapoptotic BH3-only proteins tip the balance toward cell death. At the functional level, BH3-only proteins can be divided into activators and sensitizers based on whether they bind and directly activate Bax and Bak ("activators") or antagonize antiapoptotic Bcl-2 proteins by binding to their conserved ligand binding groove ("sensitizers"), respectively (14).

Although highly debated, the origin and the evolution of the Bcl-2 protein family remain unresolved (11). Structural studies of distantly related Bcl-2 homologs identified a conserved interaction between the ligand binding groove of antiapoptotic Bcl-2 and the BH3 motif of proapoptotic multimotif proteins in sponges, albeit with subtle differences in the detailed mechanism of binding that rendered the sponge antiapoptotic Bcl-2 protein exquisitely specific for its sponge-encoded proapoptotic counterpart (15). In several animals from phylogenetically more basal branches, the repertoire of Bcl-2 family members is exclusively composed of multimotif Bcl-2 proteins, with BH3-only proteins being absent (6, 16, 17). Furthermore, Bcl-2-regulated apoptosis proceeds in the human HCT-116 cell line when all BH3-only proteins have been removed genetically (18, 19). Combined, these findings raise the possibility that the regulation of MOMP in the early stages of the metazoan evolution might be dependent solely on homo- and heteroassociation between anti and proapoptotic multimotif Bcl-2 proteins.

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ANNEX II

The Endoplasmic Reticulum pool of Bcl-xL dampens the Unfolded Protein Response through IP3R-dependent Calcium Release

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Abstract

Apoptosis plays a role in cell homeostasis in both normal development and disease. Bcl-xL, a member of the Bcl-2 family of proteins, regulates the intrinsic mitochondrial pathway of apoptosis. It is overexpressed in several cancers. Bcl-xL has a dual subcellular localization and is found at the mitochondria as well as the endoplasmic reticulum (ER). However, the biological significance of its ER localization is unclear. In order to decipher the functional contributions of the mitochondrial and reticular pools of Bcl-xL, we generated genetically modified mice expressing exclusively Bcl-xL at the ER, referred to as ER-xL, or the mitochondria, referred to as Mt-xL. By performing cell death assays, we showed that ER-xL MEFs show increased vulnerability to apoptotic stimuli but are more resistant to ER stress. Furthermore, ER-xL MEFs demonstrated a reduced expression of the Unfolded Protein Response (UPR) markers upon ER stress and displayed reduced inositol trisphosphate receptor (IP3R) mediated ER calcium release. Collectively, our data show that upon ER stress, Bcl-xL negatively regulates IP3R-mediated calcium flux from the ER, which prevents ER calcium depletion and maintains the UPR and subsequent cell death in check. This work reveals a moonlighting function of Bcl-xL at the ER, apart from its cliché regulation of apoptosis.

ANNEX III



Caspases in the Developing Central Nervous System: Apoptosis and Beyond

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The caspase family of cysteine proteases represents the executioners of programmed cell death (PCD) type I or apoptosis. For years, caspases have been known for their critical roles in shaping embryonic structures, including the development of the central nervous system (CNS). Interestingly, recent findings have suggested that aside from their roles in eliminating unnecessary neural cells, caspases are also implicated in other neurodevelopmental processes such as axon guidance, synapse formation, axon pruning, and synaptic functions. These results raise the question as to how neurons regulate this decision-making, leading either to cell death or to proper development and differentiation. This review highlights current knowledge on apoptotic and non-apoptotic functions of caspases in the developing CNS. We also discuss the molecular factors involved in the regulation of caspase-mediated roles, emphasizing the mitochondrial pathway of cell death.

Keywords: caspases, mitochondria, central nervous system, apoptosis, embryonic development

INTRODUCTION

Apoptosis is considered to be a programmed cell death (PCD) through which a multicellular organism removes damaged cells without affecting neighboring cells (Wickman et al., 2012). Apoptotic cells exhibit specific characteristics, including cell shrinkage, chromatin condensation and fragmentation, and cell membrane blebbing, followed by apoptotic body formation (Taylor et al., 2008). Shreds of evidence suggest that apoptosis, which is considered to be a self-destructive program, may also take place in healthy cells to ensure the daily functions of tissues and organs (Suzanne and Steller, 2013; Shalini et al., 2015).

The central nervous system (CNS), including the brain and spinal cord, is a complex structure that begins to appear at early stages of embryonic development. The first significant event is the formation of the neural tube from the neural plate during primary and secondary neurulation. During primary neurulation, a portion of dorsal ectoderm specifies into neural plate. Neural plate cells can be distinguished from surrounding non-neural cells by their elongated morphology. Shortly after its establishment, neural plate border thickens and rises up to create a U-shape groove. The two lateral sides of the neural groove continue to bend toward each other until their edges meet and merge to form the neural tube. Secondary neurulation then begins at the caudal end. In this latter process, that takes place in chicken and some mammalian embryos, mesenchymal cells gather to form the medullary cord under the ectoderm layer. In the next step, the medullary cord is reshaped to create a hollow cavity (Smith and Schoenwolf, 1997; Gilbert, 2000).

ANNEX IV



Review

Involvement of Bcl-xL in Neuronal Function and Development

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Abstract: The B-cell lymphoma (Bcl-2) family of proteins are mainly known for their role in the regulation of apoptosis by preventing pore formation at the mitochondrial outer membrane and subsequent caspase activation. However, Bcl-2 proteins also have non-canonical functions, independent of apoptosis. Indeed, the cell death machinery, including Bcl-2 homologs, was reported to be essential for the central nervous system (CNS), notably with respect to synaptic transmission and axon pruning. Here we focused on Bcl-xL, a close Bcl-2 homolog, which plays a major role in neuronal development, as *bclx* knock out mice prematurely die at embryonic day 13.5, showing massive apoptosis in the CNS. In addition, we present evidence that Bcl-xL fosters ATP generation by the mitochondria to fuel high energy needs by neurons, and its contribution to synaptic transmission. We discuss how Bcl-xL might control local and transient activation of caspases in neurons without causing cell death. Consistently, Bcl-xL may contribute to morphological changes, such as sprouting and retraction of axon branches, in the context of CNS plasticity. Regarding degenerative diseases and aging, a better understanding of the numerous roles of the cell death machinery in neurons may have future clinical implications.

Keywords: Bcl-xL; apoptosis; neurons; mitochondria; endoplasmic reticulum; caspases



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1. Introduction

The Bcl-2 family of proteins plays a major role in the regulation of apoptosis, a highly conserved programmed cell death process that eliminates unwanted cells, including damaged cells, which is essential during development [1]. Dysregulation of this process can lead to cancer or degenerative diseases [2,3] and multiple clinical trials aim to inhibit the Bcl-2 family proteins in cancer [4]. Bcl-2 is the founding member of this family that includes both apoptotic and non-apoptotic proteins. All of these actors are classified within three groups according to the presence of conserved Bcl-2 homology (BH) domains: the anti-apoptotic members that contain the four BH domains (BH1-4, including Bcl-2, Bcl-xL, and Mcl-1), the pro-apoptotic members, that lack the BH4 domain (including Bax and Bak), and the pro-apoptotic BH3-only proteins that contain the BH3 domain exclusively (including Bad, Bid, Bim) [5]. In addition, some proteins of the Bcl-2 family may contain a transmembrane domain in their C-terminus for their anchoring to intracellular membranes, including the mitochondrial outer membrane (MOM) and the endoplasmic reticulum (ER) [5]. BH1, BH2, and BH3 domains form a hydrophobic groove allowing anti-apoptotic Bcl-2 proteins to bind to the BH3 domain of apoptosis accelerators and thus inhibit cell death [6]. The interactions between all of these proteins determine the formation of “pores” in the MOM as a consequence of the homodimerization of the multi-domain apoptosis accelerators Bax and Bak, leading to the release of pro-apoptotic factors from the mitochondria, including cytochrome c (Figure 1). Cytochrome c release triggers the formation of the apoptosome, the complex responsible for the activation of caspases, the

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