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Control of mitochondrial physiology and cell death by the Bcl-2 family proteins Bax and Bok

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Highlights

- Bcl-2 family proteins are essential regulators of the mitochondrial apoptosis pathway.
- It is emerging that Bcl-2 proteins also have non-apoptotic, 'daytime' activities.
- Bcl-2 proteins Bax and Bok play a key role in the regulation of mitochondrial function and Ca^{2+} homeostasis in neurons.

ABSTRACT

Neuronal cell death is often triggered by events that involve intracellular increases in Ca^{2+}. Under resting conditions, the intracellular Ca^{2+} concentration is tightly controlled by a number of extrusion and sequestering mechanisms involving the plasma membrane, mitochondria, and ER. These mechanisms act to prevent a disruption of neuronal ion homeostasis. As these processes require ATP, excessive Ca^{2+} overloading may cause energy depletion, mitochondrial dysfunction, and may eventually lead to Ca^{2+}-dependent cell death. Excessive Ca^{2+} entry though glutamate receptors (excitotoxicity) has been implicated in several neurologic and chronic neurodegenerative diseases, including ischemic stroke, epilepsy, and Alzheimer's disease. Recent evidence has revealed that excitotoxic cell death is regulated by the B-cell lymphoma-2 (Bcl-2) family of proteins. Bcl-2 proteins, comprising of both pro-apoptotic and anti-apoptotic members, have been shown to not only mediate the intrinsic apoptosis pathway by controlling mitochondrial outer membrane (MOM) integrity, but to also control neuronal Ca^{2+} homeostasis and energetics. In this review, the role of Bcl-2 family proteins in the regulation of apoptosis, their expression in the central nervous system and how they control Ca^{2+}-dependent neuronal injury are summarized. We review the current knowledge on Bcl-2 family proteins in the regulation of mitochondrial function and bioenergetics, including the fusion and fission machinery, and their role in Ca^{2+} homeostasis

regulation at the mitochondria and ER. Specifically, we discuss how the 'pro-apoptotic' Bcl-2 family proteins, Bax and Bok, physiologically expressed in the nervous system, regulate such 'non-apoptotic/daytime' functions.

Keywords

Bcl-2 proteins, Mitochondria, Calcium, Excitotoxicity, Bax, Bok.

Abbreviations used in this paper

$\Delta\psi_m$, mitochondrial membrane potential; Bcl-2, B cell lymphoma gene 2; BH, Bcl-2 homology region; Bax, Bcl-2-associated protein x; Bak, Bcl-2-antagonist/killer; Mcl-1, myeloid cell leukemia gene 1; A1, Bcl-2-related protein A1; Bim, Bcl-2 interacting mediator of cell death; Puma, p53 upregulated modulator of apoptosis; Bid, BH3 interacting-domain death agonist; Bad, Bcl-2-associated death promoter; Bik, Bcl-2-interacting killer; Hrk, Harakiri; Bmf, Bcl-2-modifying factor; tBid, truncated Bid; Bok, Bcl-2-related ovarian killer; CNS, central nervous system; ER, endoplasmic reticulum; IMS, intermembrane space; MEF, mouse embryonic fibroblast; MOM, mitochondrial outer membrane; MOMP, mitochondrial outer membrane permeabilization; mPTP, mitochondrial permeability transition pore; NMDA, N-Methyl-D-aspartic acid; ROS, reactive oxygen species; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}ATPase; WT, wild-type.

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1. The Bcl-2 family of proteins in the regulation of apoptosis

The *bcl-2* (B-cell lymphoma-2) gene was first discovered in the chromosomal translocation breakpoint t(14;18) in B-cell follicular lymphomas (Tsujimoto et al., 1985), and has been intensively studied for several years regarding its implication in apoptosis, tumorigenesis, tissue homeostasis, development of autoimmune disorders and cellular responses to anti-cancer therapeutics (Delbridge and Strasser, 2015). The Bcl-2 protein family consists of a network of pro-apoptotic and anti-apoptotic members that, through their interaction with each other, function as the major regulators and effectors of the 'intrinsic' or mitochondrial apoptosis pathway. During development and in response to stress or death signals, the relative expression and activation levels of Bcl-2 proteins decide the cellular destiny, either constraining or promoting cell death execution (Tsujimoto, 2003, Danial and Korsmeyer, 2004, Youle and Strasser, 2008). According to structural and functional characteristics, the Bcl-2 family contains between one and four Bcl-2 homology (BH) domains, which play essential functions in mediating hetero- and homo-dimeric interaction among the Bcl-2 family members (Adams and Cory, 1998, Danial and Korsmeyer, 2004). Bcl-2 proteins are divided into three subfamilies: (i) the anti-apoptotic Bcl-2 family members, including Bcl-2, Bcl-xL (Bcl-extra long), A1, Bcl-w, Boo (Bcl-2 homolog of ovary) and Mcl-1 (myeloid cell leukaemia-1). These represent multidomain proteins, most of them containing all four BH (BH1, BH2, BH3, and BH4) domains. Anti-apoptotic proteins also contain a transmembrane domain (TM), that enables association with membranes including the MOM, ER, or nuclear membranes (Krajewski et al., 1993, Kroemer et al., 2007). One of the main biological functions of anti-apoptotic Bcl-2 proteins is to prevent the disruption of mitochondrial integrity. (ii) The pro-apoptotic Bcl-2 homology 3 (BH3)-only proteins, such as Bid (BH3 interacting domain death agonist), Bim (Bcl-2 interacting mediator), Bik (Bcl-2 interacting killer), Bad (Bcl-2 associated death promoter), Bmf (Bcl-2 modifying factor, Hrk (Hara-kiri),

Noxa (Latin name for “damage”) and Puma (p53 upregulated modulator of apoptosis), contain only the BH3 domain. These act as apoptosis initiators and direct antagonists of the anti-apoptotic Bcl-2 proteins; and (iii) the pro-apoptotic Bax-like subfamily, including Bax (Bcl-2 associated-x) and Bak (BH3 homologous agonist killer), and potentially Bok (Bcl-2 related ovarian killer). These three proteins contain three conserved BH domains (BH1, BH2 and BH3). Activated and oligomerized Bax and Bak form pores within the outer mitochondrial membrane that allow for the release of pro-apoptotic factors from the intermembrane space into the cytosol, a process called mitochondrial outer membrane permeabilization (MOMP) (Youle and Strasser, 2008, Chipuk et al., 2010, Czabotar et al., 2014).

In response to an apoptotic cellular stress, such as viral infections, DNA damage, ER stress or growth-factor deprivation, selected BH3-only proteins, such as Bid, Bim and Puma, activate the pro-apoptotic members Bax and/or Bak, either directly, through conformational changes (Strasser et al., 2000, Letai et al., 2002), or indirectly, through the displacement and neutralization of anti-apoptotic Bcl-2 proteins (Uren et al., 2007, Willis et al., 2007). In healthy cells, inactive Bax and Bak reside essentially in the cytosolic (Bax) or are loosely bound to the mitochondria (Bak) (Hsu et al., 1997b, Goping et al., 1998, Hsu and Youle, 1998). Following BH3-only protein activation, Bax and Bak undergo conformational changes and fully insert into the MOM, where they oligomerize and form protein-permeable channels, subsequently promoting the release of pro-apoptotic factors, such as cyt-*c*, AIF, EndoG, Smac/DIABLO and Omi, from the mitochondrial intermembrane space into the cytosol (Liu et al., 1996, Susin et al., 1996, Du et al., 2000, Kuwana et al., 2002, Kilbride and Prehn, 2013). As a result, a caspase-dependent or –independent cell death process is triggered within minutes or hours after MOMP.

2. Bcl-2 proteins in the CNS

Members of the pro- and anti-apoptotic Bcl-2 family proteins are expressed throughout the CNS during both embryonic and adult life (Lindsten et al., 2005). With regard to the pro-apoptotic, Bax-like proteins, Bax is widely expressed in the brain (Krajewski et al., 1995b), while Bok is present in the cerebral cortex and highly enriched in the CA3 subfield of the hippocampus (Lein et al., 2004, Newrzella et al., 2007, D'Orsi et al., 2016). Full-length Bak is present only in non-neuronal cells in the brain, however, studies identified a novel neuron-specific splice variant of Bak (N-Bak), that unusually only contains the BH3 domain, suggesting its potential role upstream of Bax in the cell death pathway (Sun et al., 2001, Uo et al., 2005). Although BH3-only proteins have the common characteristic of sharing the nine amino acid BH3-domain, individual members are structurally different and exhibit distinct levels of tissue expression. Some BH3-only proteins, such as Noxa and Puma, display negligible expression in healthy cells, including neurons, but can be transcriptionally activated under stress conditions. Other BH3 only protein members, including Bim, Bmf, Bad and Bid, are expressed under physiological conditions, but can be activated through post-translational modifications, including phosphorylation, intracellular displacement, or proteolytic cleavage in response to cell death signaling (Ward et al., 2004, Lomonosova and Chinnadurai, 2008, Engel et al., 2011). Of the anti-apoptotic members, Bcl-xL and Bcl-w are mostly expressed in mature neurons in the adult brain (Motoyama et al., 1995, Roth et al., 2000, O'Reilly et al., 2001), whereas Bcl-2 is widely expressed in the developing brain, particularly in sensory and sympathetic neurons (Merry et al., 1994). Mcl-1 is also expressed in the adult CNS, and is particularly enriched in neuroendocrine cells and sympathetic neurons (Krajewski et al., 1995a, Mori et al., 2004).

3. Bcl-2 proteins control neuronal injury

In the mammalian CNS, glutamate, the major neuronal excitatory neurotransmitter released by presynaptic neurons, plays a crucial role for several physiological functions, such as neurotransmission and synaptic plasticity, learning and memory, and transmission of sensory information. It also plays an important role during neuronal injury following various neurologic insults, such as ischemia, trauma and epileptic seizure. During synaptic transmission**,** glutamate activates postsynaptic ionotropic receptors, including N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and Kainate Acid (KA) receptors (Collingridge, 1994). Although physiological activation of glutamate receptors is necessary for cell survival, their pathophysiological overactivation often leads to cell death (Olney et al., 1972). In many neurons, excitotoxic injury is primarily triggered by neuronal Ca^{2+} overloading through extrasynaptic Ca^{2+}-permeable NMDA receptors (Hardingham and Bading, 2010). The best characterized and established examples of neurological conditions involving excitotoxic mechanisms are stroke and traumatic brain injury, in which glutamate release is triggered by ATP depletion or tissue trauma, as well as epileptic seizures, where excitatory synapses become overactive (Schanne et al., 1979, Choi, 1994, Zipfel et al., 1999). Excitotoxicity also plays a role in mediating chronic neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis and Huntington's disease (Rothstein et al., 1990, Bezprozvanny and Hayden, 2004, Lipton, 2007, Mattson, 2007, Mehta et al., 2013).

The downstream effects of the increased entry of Ca^{2+} in the cytosol includes energy depletion, enhanced mitochondrial stress, production of reactive oxygen species (ROS), and mitochondrial membrane depolarization. This is accompanied by the activation of a number of Ca^{2+}-dependent and catabolic processes, including the activation of proteases,

phospholipases, PARP-1 and nucleases, consequently causing cellular toxicity, neuronal dysfunction and death (Berliocchi et al., 2005, Friedman, 2006).

Several studies have shown the importance of the Bcl-2 family members in the regulation of excitotoxic cell death (Lindsten et al., 2005, Anilkumar and Prehn, 2014). Gene targeting of Bcl-2 family members in mice has provided key insights into their role in excitotoxic injury and in disease models of neurological disorders. For instance, transcriptionally or post-translationally activated BH3-only proteins, such as Bim, Puma, and Bid have been shown to mediate delayed neuronal injury (Konig et al., 2007, Steckley et al., 2007, Concannon et al., 2010). Similarly, numerous reports have demonstrated that overexpression of anti-apoptotic proteins, including *bcl-2* and *bcl-xL*, and genetic deficiency or biochemical inhibition of pro-apoptotic members, such as *bim* and *bax*, protect neurons from excitotoxic injury (Jia et al., 1996, Lawrence et al., 1996, Asoh et al., 2002, Garrity-Moses et al., 2005, Iriyama et al., 2009, D'Orsi et al., 2012, D'Orsi et al., 2015). Despite evidence that only the combined absence of *bax* and *bak* provides resistance to cell death of neuroprogenitors (D'Sa et al., 2003, Lindsten et al., 2003), several papers showed that single deletion of *bax* is sufficient to confer protection against neurotrophic growth factors deprivation, excitotoxicity, DNA damage, proteotoxic-, and oxidative stress (Deckwerth et al., 1996, Miller et al., 1997, Deshmukh and Johnson, 1998, Xiang et al., 1998, Li et al., 2004, Siu and Alway, 2006, Steckley et al., 2007, D'Orsi et al., 2015, D'Orsi et al., 2016), whereas the absence of *bak* gene failed to provide protection (Putcha et al., 2002). This lack of redundancy may be caused by the fact that full-length Bak is not expressed at detectable levels in neurons.

Increasing evidence suggest that, in addition to their role in controlling the intrinsic apoptosis pathway, Bcl-2 family proteins are also essential in the regulation of mitochondrial functions, including fusion and fission (Autret and Martin, 2010), bioenergetics (Alavian et al., 2011, Jonas et al., 2014, D'Orsi et al., 2015, D'Orsi et al., 2016), and neuronal Ca^{2+} handling (Pinton

et al., 2000, Chen et al., 2004, Oakes et al., 2005, D'Orsi et al., 2015, D'Orsi et al., 2016), activities representing their "daytime"/non-apoptotic functions (Kilbride and Prehn, 2013). In the remainder of this review, we focus on the emerging role of Bcl-2 family proteins in mitochondrial and calcium functions, particularly concentrating on the involvement of Bax and Bok in controlling cell death.

4. Bcl-2 proteins in the regulation of mitochondrial function

Mitochondria are highly dynamic organelles that actively interact with mitochondria of neighbouring cells and other intracellular compartments to sustain energy metabolism, rapidly repair damaged mitochondria and exchange cellular components, such as DNA and proteins. Neurons are principally dependent on mitochondria for energy production, in the form of ATP through oxidative phosphorylation, and for the regulation of several physiological functions, including cell proliferation, neurotransmission and intracellular calcium buffering (van Belzen et al., 1997, Nicholls and Budd, 2000, Brookes et al., 2004). Therefore, neurons are highly sensitive to alterations of mitochondrial function, which may result in dysfunctional synapses, axonal degeneration, and eventually cell death. Mitochondria, through a tightly controlled process involving Ca^{2+}-sensitive dehydrogenases, the mitochondrial calcium uniporter (MCU) and the Na^{+}/Ca^{2+} exchanger, regulate intracellular Ca^{2+} levels in cytosol and control the extent, position and propagation of cytosolic Ca^{2+} influx and its recycle towards the ER (Grienberger and Konnerth, 2012, Rizzuto et al., 2012, Lopreiato et al., 2014). Ca^{2+} overloading, however, as seen during intense overactivation of glutamate receptors leads to subsequent mitochondrial dysfunction and neuronal injury (Chen and Chan, 2009, Su et al., 2010). Several Bcl-2 family proteins reside at, or translocate to, the mitochondria and are believed to play a role also in normal

mitochondrial physiology. The observations that apoptosis is accompanied by mitochondrial fragmentation and this can often coincide with MOMP and cyt-*c* release provided indication of a cross-talk between the Bcl-2 family functions and the regulation of mitochondrial dynamics (Frank et al., 2001, Karbowski et al., 2002, Perfettini et al., 2005, Autret and Martin, 2009). Indeed, during apoptosis, components of the mitochondrial fusion and fission machinery, such as Drp1 and Mfn2, are recruited to mitochondrial scission sites and colocalize with Bax, promoting a Drp1-dependent mitochondrial fragmentation (Frank et al., 2001, Karbowski et al., 2002, Brooks et al., 2007, Wasiak et al., 2007). Moreover, Bax and/or Bak induce mitochondrial network fragmentation, though the release of the IMS protein DDP/TIMM8a into the cytoplasm, promoting Drp1 redistribution to the mitochondria and activating Drp1-mediated fission (Karbowski et al., 2002, Arnoult et al., 2005, Sheridan et al., 2008). It has also been demonstrated that inhibition of the fission process by downregulating Fis1 or Drp1, alters cyt *c* release and delays apoptosis, suggesting a connection between Drp1-mediated mitochondrial fission, Bax-dependent MOMP and cyt *c* release (Frank et al., 2001, Breckenridge et al., 2003, Lee et al., 2004, Suen et al., 2008). Furthermore, the mitochondrial Drp1 inhibitor Mdivi-1 blocks Bid-mediated Bax/Bak-dependent cyt *c* release from mitochondria (Cassidy-Stone et al., 2008). However, Bcl-2 protein also directly regulate mitochondrial fusion and fission independent of mitochondrial apoptosis engagement. For example, *bax* and *bak* gene deletion resulted in a reduction in mitochondrial fusion in mouse embryonic fibroblasts (MEF) (Karbowski et al., 2006) whilst they were protected from apoptosis, whereas overexpression of Bax and Bak alone was able to trigger mitochondrial fission (Sheridan et al., 2008). Therefore, an increase in Bax and Bak at the MOM, or changes in the ratio of pro-apoptotic Bax and Bak *versus* anti-apoptotic Bcl-2 family members may influence not only cell death signaling, but also mitochondrial morphology, with increased Bax and Bak signaling causing mitochondrial fragmentation or

inhibition of mitochondrial fusion (Rolland and Conradt, 2010). Bax and Bak are not the only Bcl-2 proteins linked to mitochondrial dynamics. Recent studies showed that Bcl-2 and Bcl-xL associate with Mfn2 and promote mitochondrial fusion (Chipuk et al., 2010). Pro-apoptotic BH3-only proteins, such as Noxa and Puma, have also been implicated in triggering Drp1-dependent mitochondrial fragmentation, albeit during apoptosis (Sheridan et al., 2008, Woo et al., 2009). Lately, a new study revealed that the switch from the anti-apoptotic Mcl-1 long isoform (Mcl-1 L), which binds to Drp1 to promote fission and prevent apoptosis, to the pro-apoptotic Mcl-1 short isoform (Mcl-1 S) caused mitochondrial hyperfusion and hyperpolarization, resulting in increased mitochondrial Ca^{2+} accumulation and sensitivity to apoptotic stress (Morciano et al., 2016).

Mitochondrial dysfunction is also associated with a fragmentation of the mitochondrial network and remodeling of cristae, characterized by fusion of individual cristae and widening of the cristae junctions, resulting in the removal of the diffusion barrier and mobilization of cyt-*c* from intra cristae to the intermembrane space (IMS) (Frank et al., 2001, Scorrano et al., 2002). Several proteins, involved in mitochondrial fusion/fission dynamics may also play a crucial role in the pro-apoptotic remodeling of cristae. Of note, Drp1 is required for the optimal release of cyt-*c*, likely through its contribution to cristae remodeling (Germain et al., 2005). Other studies suggested that Opa1 contributes to maintain the cristae structure and its proteolytic activation causes mitochondrial fragmentation and alters the cristae shape (Olichon et al., 2003). Upon apoptosis induction, the BH3-only proteins, Bid and Bik, have been shown to disrupt Opa1 oligomers, causing rearrangements of the sub-mitochondrial structure and loss of their compartmentalization, leading to cyt-*c* mobilization, MOMP and release of IMS proteins (Scorrano et al., 2002, Germain et al., 2005). In recent years, Bid has also been involved in mitochondrial metabolism, as it acts as upstream negative regulator of the mitochondrial carrier homolog 2 (Mtch2), a protein implicated in reducing mitochondrial

diameter and metabolism. Mtch2 has been suggested to enable Bid to target to the mitochondria, functioning as a mitochondrial receptor-like protein, and accelerates the activation of the downstream pro-apoptotic protein Bax and Bak (Zaltsman et al., 2010, Shamas-Din et al., 2013, Maryanovich et al., 2015).

The Bcl-2 family of proteins have also been proposed to control the opening of mitochondrial permeability transition pore (mPTP), located between the outer and the inner mitochondrial membranes, a protein complex thought to be composed of the adenine-nucleotide translocator (ANT), the voltage-dependent anion channel (VDAC) and the modulatory protein Cyclophilin D (CypD). Evidence has been presented that under prolonged excessive Ca^{2+} influx into the mitochondrial matrix, ANT, VDAC and CypD form a protein complex that allows for the release of matrix constituents and is associated with necrotic cell death (Halestrap, 2009, Bernardi and von Stockum, 2012). Anti-apoptotic Bcl-2 family members, including Bcl-2 and Bcl-xL, have been suggested to modulate the mPTP by maintaining it in a close conformation, while pro-apoptotic proteins, such as Bax and Bak, engage ANT and/or VDAC to induce mPTP opening (Marzo et al., 1998, Shimizu et al., 1999, Whelan et al., 2012, Karch et al., 2013). However, other studies demonstrated that ANT and VDAC are dispensable for both mPTP and Bcl-2 family proteins-controlled cell death (Kokoszka et al., 2004, Baines et al., 2007). More recently, it has been suggested that opening of the PT pore under stress may also be regulated by Bcl-xL and its binding to the mitochondrial β-subunit of the F0/F1-ATP synthase. It has been proposed that the F0/F1-ATP synthase, through its c-subunit also acts as the core component of the mPTP (Chen et al., 2011, Bonora et al., 2013, Giorgio et al., 2013, Alavian et al., 2014, Jonas et al., 2014).

5. Anti-apoptotic Bcl-2 proteins and Ca^{2+} signaling

Mitochondria not only interact with themselves but also with the ER within neurons. Importantly, Bcl-2 family protein also localize at the ER, possibly modulating the interaction between mitochondria and ER, by regulating ER calcium stores and signaling in non-neuronal cells (Rong and Distelhorst, 2008, Chipuk et al., 2010). Selected Bcl-2 proteins have been shown to influence Ca^{2+} accumulation in the ER by modulating the expression and/or activity of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA), and the mobilization of Ca^{2+} from the ER to the cytosol or mitochondria, by mediating the opening of inositol 1,4,5-triphosphate receptors (IP_3Rs). It has long been clear that Bcl-2 suppresses Ca^{2+} release from the ER, however, the mechanism is being debated by partially contradictory studies. For instance, it has been shown that Bcl-2 reduced resting ER Ca^{2+} levels and cytosolic Ca^{2+} oscillations in HeLa and MEF cells (Pinton et al., 2000, Rong et al., 2009). Nevertheless, other studies demonstrated that Bcl-2 inhibited ER Ca^{2+} release dynamics without having an effect on ER Ca^{2+} store (Lam et al., 1994, Distelhorst et al., 1996, He et al., 1997, Wang et al., 2001, Chen et al., 2004). Bcl-2 regulates ER Ca^{2+} pools, where its overexpression reduced ER and Golgi Ca^{2+} stores loading (Foyouzi-Youssefi et al., 2000, Pinton et al., 2000) and Bcl-2 silencing or SERCA overexpression restored ER Ca^{2+} (Scorrano et al., 2003, Oakes et al., 2005). In addition, Bcl-2, Bcl-X_L and Mcl-1 promote pro-survival Ca^{2+} oscillations by regulating the IP_3Rs (White et al., 2005, Eckenrode et al., 2010, Monaco et al., 2012), where in particular, Bcl-xL affects thapsigargin-induced Ca^{2+} dynamics by altering the expression of IP3Rs levels (Li et al., 2002). Mcl-1 overexpression in neurons reduces cytosolic Ca^{2+} overloading in response to NMDA excitation (Anilkumar et al., 2013). The anti-apoptotic proteins Bcl-2, Bcl-X_L and Mcl-1 all regulate mitochondrial Ca^{2+} uptake through the interaction with VDAC1 (Shimizu et al., 1999, Arbel and Shoshan-Barmatz, 2010, Arbel et al., 2012, Huang et al., 2013, Huang et al., 2014), while Bcl-2 has been shown

to affect Ca^{2+} extrusion both at mitochondrial and cellular level. For instance, Bcl-2 inhibits the mitochondrial Na^{+}/Ca^{2+} exchanger activity, reducing the extrusion of Ca^{2+} from the mitochondrial matrix (Zhu et al., 2001) and controls the plasma membrane Ca^{2+} ATPase (PMCA), either inhibiting or amplifying its activity based on Bcl-2 increasing or decreasing levels, respectively (Ferdek et al., 2012). Ryanodine receptors (RyRs) at the ER have been classified as a target of Bcl-2 and Bcl-xL, in which the BH4 domain has been implicated to inhibit the RyR-mediated Ca^{2+} release in overexpression cell models and also dissociated hippocampal neurons (Vervliet et al., 2014, Vervliet et al., 2015a, Vervliet et al., 2015b). Moreover, Bax and Bak had also been suggested to modulate ER Ca^{2+} stores, possibly by inactivating the inhibitory functions of Bcl-2 and Bcl-xL on the IP3Rs (Scorrano et al., 2003, Oakes et al., 2005), and Bok has been implicated in the upregulation of the IP3Rs by protecting them from proteolysis (Schulman et al., 2013, Schulman et al., 2016). The main findings on the control of Ca^{2+} signaling by anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1) and pro-apoptotic (Bax and Bok) Bcl-2 family proteins, for both neuronal and non-neuronal cells, are discussed below and summarized in Table 1.

Table 1. Bcl-2 family proteins in Ca^{2+} signaling

Protein	Cell Death Function	Model system	'Day time' function in Ca^{2+} signaling	References
Bcl-2	anti-apoptotic	HeLa, MEF, R6, HEK293, WEHI7.2 cells	Reduces ER, Golgi and intracellular Ca^{2+} loading	(Pinton et al., 2000, Rong et al., 2009)
		WEHI7.2, HEK293 cells	Inhibits ER Ca^{2+} release dynamics with no effects on ER Ca^{2+} stores	(Lam et al., 1994, Distelhorst et al., 1996, He et al., 1997, Wang et al., 2001, Chen et al., 2004)
		MEF cells	When silenced, ER Ca^{2+} is restored	(Scorrano et al., 2003, Oakes et al., 2005)
		DT40 cells	Increases the rate of InsP(3)-mediated Ca^{2+} release	(Eckenrode et al., 2010)
		T-REx-293 cells	Intensifies mitochondrial Ca^{2+} uptake through VDAC1 interaction	(Arbel and Shoshan-Barmatz, 2010)

		Mouse heart	Reduces the extrusion of Ca^{2+} from the mitochondrial matrix	(Zhu et al., 2001)
		Mouse Pancreatic acinar cells	Inhibits (increased Bcl-2 levels) or amplifies (decreased Bcl-2 levels) the PMCA activity	(Ferdek et al., 2012)
		HEK293 cells, hippocampal neurons	Inhibits RyR-mediated Ca^{2+} release	(Vervliet et al., 2014, Vervliet et al., 2015b)
Bcl-X_L	anti-apoptotic	DT40 cells	Enhances IP_3Rs-dependent ER Ca^{2+} signaling	(White et al., 2005)
		2B4.11 murine T cells	Reduces Thapsigargin-induced Ca^{2+} flux, decreases IP3Rs expression levels	(Li et al., 2002)
		MEF, HepG2, T-REx-293cells, rat liver mitochondria	Improves mitochondrial Ca^{2+} uptake through VDAC1interaction	(Shimizu et al., 1999, Arbel et al., 2012, Huang et al., 2013)
		HEK293 cells, Hippocampal neurons	Inhibits RyR-mediated Ca^{2+} release	(Vervliet et al., 2015a)
Mcl-1	anti-apoptotic	DT40 cells	Enhances the rate of InsP(3)-mediated Ca^{2+} release	(Eckenrode et al., 2010)
		Cortical neurons	When overexpressed, cytosolic Ca^{2+} overloading is reduced	(Anilkumar et al., 2013)
		A549 cells	Limits mitochondrial Ca^{2+} uptake through VDAC1 interaction	(Huang et al., 2014)
Bax	pro-apoptotic	DU-145, PC-3 cells	When overexpressed, the transfer of Ca^{2+} from ER to mitochondria is facilitated	(Nutt et al., 2002a, Nutt et al., 2002b)
		MEF cells	When deleted, ER Ca^{2+} release is reduced	(Scorrano et al., 2003)
		Cortical neurons	Facilitates Ca^{2+} signaling between ER and cytosol	(D'Orsi et al., 2015)
		Cortical neurons	When deleted, Ca^{2+} transients are reduced	(D'Orsi et al., 2015)
Bok	anti-apoptotic or neutral effect in studies using gene deficient neurons; tissue-specific pro-apoptotic, anti-apoptotic or neutral effects in other tissues	Cortical neurons	When deleted, Ca^{2+} homeostasis is decreased	(D'Orsi et al., 2016)
		MEF cells	When deleted, ER stress is increased, possibly through a Ca^{2+} release mechanism	(Echeverry et al., 2013, Fernandez-Marrero et al., 2016, Llambi et al., 2016)

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6. Bax and Ca^{2+} signaling

bax was the first Bcl-2 homologue gene to be identified acting as an apoptosis executer. Bax protein is expressed in various tissues, as multiple alternative splice variants, normally localized in the cytosol or loosely attached to the mitochondria. The best characterized isoform is the 21 kDa Bax*α* which contains three BH domains and membrane anchor domains, allowing for insertion in the mitochondria upon apoptosis stimulation (Wolter et al., 1997, Zhou et al., 1998). *bax* gene deletion has been demonstrated to grant neuroprotection against several apoptotic stimuli *in vitro*, including neurotrophic factor deprivation, excitotoxic and DNA damage-induced cell death (Deckwerth et al., 1996, Miller et al., 1997, Deshmukh and Johnson, 1998, Xiang et al., 1998, Wang et al., 2004, D'Orsi et al., 2015, D'Orsi et al., 2016), and in models of excitotoxic/ischemic injury *in vivo* (Perez-Navarro et al., 2005, D'Orsi et al., 2015). As alluded to above, Bax, apart from its role in regulating the intrinsic apoptosis pathway, plays key functions in mitochondrial bioenergetics and Ca^{2+} homeostasis (Nutt et al., 2002a; Nutt et al., 2002b; Scorrano et al., 2003; Chami et al., 2004; D'Orsi et al., 2015; D'Orsi et al., 2016). There is strong evidence that Bax directly modifies Ca^{2+} dynamics independent of its putative pore-forming region. In fact, Bax expression in HeLa cells resulted in an increased ER Ca^{2+} loading, followed by release of Ca^{2+} from the ER, an increase in mitochondrial Ca^{2+} loading and potentiation of mitochondrial Ca^{2+} responses, consequently triggering apoptosis (Chami et al., 2004). These results were in line with previous studies in which Bax/Bak overexpression was reported to facilitate the transfer of Ca^{2+} from ER to mitochondria, sensitizing the mitochondria to absorb more Ca^{2+}, thereby inducing cell death (Nutt et al., 2002a, Nutt et al., 2002b). Both *bax*- and *bad*-deficient mice and Bax/Bak DKO MEF cells displayed decreased ER Ca^{2+} stores, resulting in a reduction in ER Ca^{2+} release and a resistance to a wide range of apoptotic stimuli, including ceramide, staurosporine, arachidonic acid and H_2O_2 (Scorrano et al., 2003). Bax also regulates the

dynamic Ca^{2+} signaling between ER and cytosol in cortical neurons, independently from its classical function in the apoptotic cell death machinery or a proposed involvement in mitochondrial PTP opening (D'Orsi et al., 2015). Neurons lacking *bax* exhibited significantly reduced Ca^{2+} transients and deregulation of the mitochondrial membrane potential ($\Delta\psi_m$) in response to NMDA-induced excitotoxicity compared to their WT controls. Altered ER Ca^{2+} handling was also observed when inhibition of Ca^{2+} uptake into the ER was accomplished using the SERCA inhibitor, Thapsigargin (D'Orsi et al., 2015). The study also demonstrated that any effects of Bax on mPTP opening in intact cells may be secondary to the effects of Bax on cytosolic Ca^{2+} handling, and tested the hypothesis that Bax may directly or indirectly control mitochondrial energetics. However, *bax* deficiency did not improve neuronal bioenergetics and slightly reduced basal cytosolic ATP levels (D'Orsi et al., 2015).

7. Bok and Ca^{2+} signaling

Bok is expressed in hippocampal (including CA3) and cortical neurons (Lein et al., 2004, Newrzella et al., 2007, D'Orsi et al., 2016). Due to its predicted structural homology to the pro-apoptotic Bcl-2 members Bax and Bak, Bok has been proposed to act in a similar pro-apoptotic pathway (Hsu et al., 1997a, Inohara et al., 1998, Bartholomeusz et al., 2006, Rodriguez et al., 2006). So far, there are limited reports on the role of Bok in neuronal injury. However, a recent study provided new insights into the functional role of Bok during neuronal apoptosis and Ca^{2+}-mediated neuronal injury, demonstrating that, contrary to previous proposals, Bok exerts neuroprotective activities *in vitro* and *in vivo* (D'Orsi et al., 2016). Bok was first identified in a yeast two-hybrid screening using Bcl-2 anti-apoptotic members as baits, where it strongly interacted with Mcl-1, BHRF1, and Bfl-1, but not with Bcl-2, Bcl-xL and Bcl-w (Hsu et al., 1997a, Inohara et al., 1998). Overexpression of Bok has

been shown to promote cyt-*c* release, caspase-3 activation, nuclear fragmentation and apoptosis in several mammalian cell models (Inohara et al., 1998, Igaki et al., 2000, Zhang et al., 2000, Yakovlev et al., 2004, Bartholomeusz et al., 2006). Furthermore, *bok* gene silencing is seen in some human cancers, suggesting a potential role as tumor suppressor (Beroukhim et al., 2010). Bok has recently been attributed a pro-apoptotic role in regulating ER- and proteasome stress-induced apoptosis, where Bok promotes MOMP independently of Bax, Bak and activator BH3-only peptides, and its expression leads to a ER-associated degradation (ERAD) pathway-dependent cell death (Einsele-Scholz et al., 2016, Llambi et al., 2016). Others provided evidence that C-terminally truncated recombinant Bok ($Bok^{\Delta C}$) permeabilizes liposomes and cooperates with tBid in forming large and stable pores in artificial membranes that mimic mitochondrial membranes (Fernandez-Marrero et al., 2016). However, single gene *bok* or double *bax/bok* and *bak/bok* deletions in mice showed normal morphological or functional development (Ke et al., 2012, Ke et al., 2013). In contrast, *bax*/*bak* double knockout mice displayed numerous phenotypic abnormalities affecting their ability to reach adult life (Lindsten et al., 2000, Wei et al., 2001), suggesting that Bok is not capable to compensate entirely for a *bax* and/or *bak* loss. Other studies in non-neuronal cells also provided evidence that Bok possesses non-apoptotic functions in the regulation of trophoblast cell proliferation (Ray et al., 2010) or even to have pro-survival rather than a pro-death role (Echeverry et al., 2013, D'Orsi et al., 2016). We and others showed that deletion of *bok* failed to protect mouse cortical neurons and hematopoietic cells against several apoptosis-inducing stimuli (Ke et al., 2012, Echeverry et al., 2013, D'Orsi et al., 2016). Similarly, Bok was not required for STS-, etoposide- and UV-induced apoptosis in MEF cells (Carpio et al., 2015). Nevertheless, the physiological or pathophysiological role of Bok still remains controversial and it is possible that the Bok redundancy with Bax and Bak may be tissue-related (Ke et al., 2015).

Bok localizes to various cellular organelles, although, the atypical C-terminal transmembrane domain of Bok has higher affinity for the ER and Golgi membranes than to the mitochondria (Echeverry et al., 2013). At the ER, Bok binds strongly and constitutively to IP_3R1 and IP_3R2, regulating their protein levels and protecting them from proteolytic cleavage and caspase-mediated degradation (Schulman et al., 2013). All cellular Bok is IP_3Rs bound and unbound Bok becomes ubiquitinated and rapidly degraded by the proteasome (Schulman et al., 2016). Previous studies also suggested that *bok* deficiency produced increased ER stress, possibly through a Ca^{2+} release mechanism (Echeverry et al., 2013, Fernandez-Marrero et al., 2016, Llambi et al., 2016). In neurons, a recent report demonstrated that *bok*-deficient cortical neurons exhibited significantly increased neuronal injury in models of NMDA-, OGD- and seizure-induced cell death *in vitro* and *in vivo* (D'Orsi et al., 2016). *bok*-deficient neurons failed to maintain their neuronal Ca^{2+} homeostasis and showed reduced mitochondrial energetics and increased PARP-1 activation in response to excitotoxicity. Moreover, *bok* deficiency also led to a specific reduction in neuronal Mcl-1 protein levels, and both mitochondrial bioenergetics and Ca^{2+} handling defects were rescued by Mcl-1 overexpression, suggesting that the combined presence of Bok and Mcl-1 was required for the maintenance of mitochondrial energetics (D'Orsi et al., 2016).

8. Conclusions

In this review, we have discussed several mechanisms by which the Bcl-2 proteins control mitochondrial and Ca^{2+} dynamics and how these relate to neuronal cell death. Emerging studies suggest that pro- and anti-apoptotic members of the Bcl-2 protein family not only modulate the mitochondrial pathway of apoptosis, but also possess important ‘day-time’ activities. These functions include the regulation of neuronal Ca^{2+} homeostasis and mitochondrial energetics. Therefore, a better understanding of physiological and

pathophysiological role these proteins may be beneficial for future studies considering Bcl-2 proteins as therapeutic targets for the treatment of neuronal injury. Successful targeting of Bcl-2 has already been achieved. Bcl-2 antagonists as apoptosis sensitizers have recently progressed to clinical trials in the form of the selective Bcl-2 antagonist, ABT-199 or Venetoclax. Venetoclax has been approved for the treatment of chronic lymphocytic leukemia, as it has been shown to enhance death of tumor cells (Roberts et al., 2016, Stilgenbauer et al., 2016). The development of inhibitors of pro-apoptotic Bcl-2 family proteins, such as Bax antagonists, or of Bcl-2 agonists is less advanced. Development of such targeted therapeutics will not only allow new insights into disease pathology, but could also deliver a novel class of neurotherapeutics.

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