PERSPECTIVES

# Mitochondrial membrane protein Bcl-xL, a regulator of adult neuronal growth and synaptic plasticity: multiple functions beyond apoptosis

The B-cell lymphoma 2 (Bcl2) family of proteins participates in cell death or survival through a mitochondrial pathway. The pro-apoptotic members of the Bcl2 family such as Bim, Bid, Bax and Bak trigger cell death by contributing to the enhancement of mitochondrial outer membrane permeability to pro-apoptotic factors such as cytochrome c, with the subsequent activation of caspases. The anti-apoptotic members, such as B-cell lymphoma-extra large (Bcl-xL), block the pro-apoptotic Bcl2 members and prevent cell death. Bcl-xL is abundantly expressed during development and in mature neurons, suggesting that it plays a role in protection from death from untoward events occurring in adult life such as ischemia, inflammation or trauma. When these neurotoxic insults occur, Bcl-xL translocates to mitochondria and prevents activation and homo-oligomerization of pro-apoptotic family members such Bax and Bak. Numerous studies have shown pro-survival roles for Bcl-xL in adult neurons using various models; nevertheless, the role of Bcl-xL outside of the field of neuronal death, *i.e.*, in adult neuronal growth, excitability or synaptic plasticity, has not been studied in depth.

Our laboratory has been investigating the function of Bcl-xL not only in mitochondrially mediated apoptotic signaling, but also in cells not exposed to death stimuli. We observed that blocking Bcl-xL does not cause immediate cell death, but rather impairs mitochondrial localization to synapses and synapse formation (Li et al., 2008). We found further that Bcl-xL improves metabolic efficiency by interacting with the $F_1F_O$ ATP synthase in the mitochondrial inner membrane, and ultimately helps neurons to produce more ATP while using less oxygen (Alavian et al., 2011; Chen et al., 2011). We also reported that Bcl-xL binds directly with dynamin-related protein 1 (Drp1). This protein-protein interaction occurs not only at mitochondrial membranes to regulate mitochondrial targeting, but also at clathrin-coated pits in the plasma membrane. Interaction of Bcl-xL with synaptic vesicle membranes is critical for the normal process of synaptic vesicle endocytosis during neuronal stimulation (Li et al., 2013).

The most recent study from our laboratory reported that Bcl-xL is necessary for neuronal outgrowth and neuronal network formation (Park et al., 2014). We applied an RNAi gene silencing technique using recombinant adeno-associated virus (rAAV) to primary hippocampal neurons. Bcl-xL-mRNA-targeting rAAV delivery consistently showed that depletion of Bcl-xL did not influence immediate neuronal death or survival. However, we observed striking differences in the morphology of Bcl-xL siRNA-expressing neurons. Bcl-xL-depleted neurons failed to elongate; in addition, neuritic branching was impaired. These changes led to very delayed death of neuronal somata after 4 weeks in culture. The morphological changes of the neurites also increased the vulnerability of neurons to neurotoxic stimuli (hypoxia).

Bcl-xL may target multiple pathways to regulate neurite outgrowth, and we identified death receptor 6 (DR6) as one molecule under Bcl-xL control. DR6, a member of the tumor necrosis factor (TNF) receptor superfamily that contains an intracellular death domain, is reported to be necessary for normal axonal pruning in response to neuronal growth factor (NGF) withdrawal in spinal neurons (Nikolaev et al., 2009). During preliminary screening, we observed upregulation of DR6 in the CA1 region of rat hippocampus after four vessel occlusion (4VO)-induced ischemia. DR6 expression was significantly upregulated at 24 hours after reperfusion then decreased at later time points. 10 minute occlusion by 4VO does not cause immediate cell death but instead results in delayed death in CA1 hippocampal neurons. Degenerated neurons typically appear at 6–7 days, but not at 24 hours after 4VO procedure. This gave us a clue that DR6 may be an ischemia-activated molecule that could trigger acute neuronal changes followed by delayed somatic death at 6–7 days.

We consistently observed upregulation of DR6 after hypoxic stimuli *in vitro*. We confirmed that DR6 is a necessary downstream effector of Bcl-xL by using the RNAi approach. Both hypoxia and Bcl-xL depletion significantly elevated DR6 expression in primary hippocampal neurons, indicating that hypoxia may induce DR6 protein expression after sequestration, or proteolytic cleavage, of Bcl-xL. We further tested the role of DR6 in neurite loss. DR6 depletion partially reversed the neurite damage that occurred in Bcl-xL depleted neurons in both normoxia and hypoxia. However, we failed to achieve complete neuroprotection by blocking DR6 with siRNA transduction, indicating that Bcl-xL may have multiple targets that regulate neuronal outgrowth and survival. We are currently investigating other neuritogenetic or neurotoxic molecules under the control of Bcl-xL.

Although Bcl-xL regulation of neurite outgrowth *via* DR6 is still under investigation, we also find that depletion of Bcl-xL using the RNAi approach activates pro-apoptotic Bax. Activated Bax is known to oligomerize at mitochondria to compromise outer membrane integrity, release caspases and activate other downstream targets, possibly further compromising neurite extension or neuronal target-seeking activities. How these caspase-dependent molecules arrest neurite growth is not yet shown.

We have shown that depletion of Bcl-xL shortens neurites and reduces neurite branching. This impairs normal neuronal network development over time. We suggest that failure of neurons to contact partner neurons eventually leads to neuronal death without necessarily implicating any exogenous neurotoxic insult, perhaps because unused synaptic connections signal death of the cell soma in a delayed fashion. Neurites may be more susceptible than somata to loss of growth factor or other signals, and losing the integrity of neurites is therefore an earlier event than somatic death. In keeping with this, neurite loss may be an isolated event not followed by somatic death in scenarios of normal synapse pruning. Neurites have high energy demands due to their role in the release and recovery of neurotransmitter pools, receptor trafficking and the navigation and extension of neurite tips at sites of formation

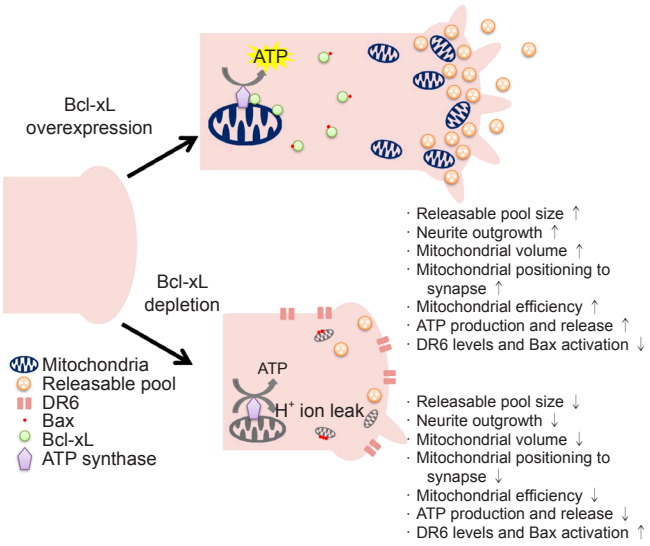


**Figure 1 Model of Bcl-xL regulation of normal neuronal properties.**
Bcl-xL enhances the efficiency of the $F_1F_O$ ATP synthase, and increases ATP production. Bcl-xL increases mitochondrial biogenesis and targeting of mitochondria to synapses. Bcl-xL enhances synaptic transmission and recovery of synaptic vesicle pools. The absence of Bcl-xL leads to loss of mitochondrial efficiency, loss of protection from pro-death factors including Bax and DR6, loss of neurite outgrowth and decreased synapse formation. Delayed cell death may eventually occur after prolonged decrease in Bcl-xL functions.

of new synapses. The dynamic nature of neurites produces high levels of reactive oxygen species (ROS) (Valencia et al., 2013). Since mitochondria are the center of energy metabolism and ROS production, it is therefore not surprising that mitochondrial dysfunction may play an early role in neurite degeneration; therefore maintaining mitochondria may be critical to neurite health (Court and Coleman, 2012).

Synapses are also major consumers of neuronal energy. ATP is required to reset ion gradients after action potential firing, to refill vesicle pools after synaptic transmission, and to maintain the resting potential (Attwell and Laughlin, 2001; Harris et al., 2012). Failure to maintain cellular ATP may be fatal for neurite outgrowth. We previously reported that genetic or pharmacological inhibition of Bcl-xL activity decreased efficiency of energy metabolism in mitochondria by increasing oxygen consumption and $H^+$ ion leak, thus decreasing ATP production. We also found that Bcl-xL binds directly to the β-subunit of the $F_1F_O$ ATP synthase to maintain or improve ATP synthase activity (Alavian et al., 2011). In contrast to depletion, overexpression of Bcl-xL enhances synapse formation in both axonal and dendritic compartments (Li et al., 2008). Therefore, the role of Bcl-xL in mitochondrial positioning and metabolism may be critical for neurite outgrowth. Mitochondria may respond to neuronal growth factors or intracellular signals and relocate to the site of neurite outgrowth to provide energy for ongoing neuritogenesis and synaptic activity. It seems natural that during this process, the absence of Bcl-xL could disrupt mitochondrial ATP homeostasis and disturb synapse formation followed by arrest of neurite extension and eventual somatic loss (Li et al., 2008; Alavian et al., 2011; Park et al., 2014) (**Figure 1**). This could have implications for neurodegenerative disease (Calkins et al., 2011). The reports from our laboratory consistently support the idea that Bcl-xL is not a simple anti-apoptotic molecule. We want to emphasize that Bcl-xL has multiple functions in energy metabolism, synaptic transmission and formation, and neuronal outgrowth in addition to its involvement in the apoptotic pathway. Although current studies on the non-apoptotic role of Bcl-xL are still in early stages, it may be time to start questioning our paradigm. Is Bcl-xL simply an anti-apoptotic molecule, or does the survival role of Bcl-xL include the maintenance and enhancement of normal neuronal properties?

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