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MINIREVIEW

Post-ischemic brain damage: NF-κB dimer heterogeneity as a molecular determinant of neuron vulnerability

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Nuclear factor-kappaB (NF-κB) has been proposed to serve a dual function as a regulator of neuron survival in pathological conditions associated with neurodegeneration. NF-κB is a transcription family of factors comprising five different proteins, namely p50, RelA/p65, c-Rel, RelB and p52, which can combine differently to form active dimers in response to external stimuli. Recent research shows that diverse NF-κB dimers lead to cell death or cell survival in neurons exposed to ischemic injury. While the p50/p65 dimer participates in the pathogenesis of post-ischemic injury by inducing pro-apoptotic gene expression, c-Rel-containing dimers increase neuron resistance to ischemia by inducing anti-apoptotic gene transcription. We present, in this report, the latest findings and consider the therapeutic potential of targeting different NF-κB dimers to limit ischemia-associated neurodegeneration.

Stroke is the third major cause of death and long-term disability in most developed countries, with very limited chance for effective treatments. The mechanisms that trigger ischemic brain damage include a plethora of biochemical and cellular events, such as glutamate-mediated excitotoxicity, generation of reactive oxygen species, DNA damage and inflammation. In focal ischemia, primary neuronal death appears rapidly in the core area and is followed by secondary death in the ischemic penumbra that evolves from the delayed activation of multiple death pathways. Thus, the infarct takes several days to mature and recruits a myriad of processes that, depending on the threshold of single neuron vulnerability, determine the final damage entity. Identifying the factors setting the limit of neuronal resistance will disclose new targets for minimizing development of the lesion. A large repertoire of genes is activated by transcription factors induced in brain ischemia, including hypoxia inducible factor-1, p53, interferon regulatory factor-1 activating transcription factor-2, signal transducer and activator of transcription 3 and nuclear factor-kappaB (NF-κB) [1]. NF-κB is a key regulator of both inflammation and cell death and has been proposed as suitable target for

Abbreviations
Bcl-2, B-cell lymphoma 2; IKK, IκB kinase; IL, interleukin; IκB, κB inhibitory protein; LTD, long-term depression; MCAO, middle cerebral artery occlusion; MEK, mitogen-activated protein kinase kinase; mGlu, metabotropic glutamate; NF-κB, nuclear factor-kappaB; NMDA, *N*-methyl-D-aspartate; OGD, oxygen-glucose deprivation; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; RHD, Rel-homology domain; TNFR, tumor necrosis factor receptor; TWEAK, tumor necrosis factor-like weak inducer of apoptosis.

the treatment of brain ischemia [1–3]. In this report we review the most recent advances in understanding the mechanisms responsible for the dual effect produced by NF-κB in the post-ischemic injury and for predicting possible new experimental approaches.

Molecular activation of NF-κB

In the past two decades, much work focused on NF-κB family proteins has proposed this ubiquitously expressed transcription factor as a pleiotropic regulator of target genes controlling physiological function in the nervous system. In mammals, the NF-κB family comprises five members, sharing an N-terminal 300 amino acid Rel-homology domain (RHD), which is identical in 35–61% of all NF-κB family proteins: p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100, which are encoded by *RELA*, *RELB*, *REL*, *NFKB1* and *NFKB2* respectively. The RHD domain allows dimerization, nuclear translocation and DNA binding. Among the members of the NF-κB family, only p65, c-Rel and RelB are directly able to activate the transcription of target genes. The transcriptional capacities of p50 and p52, which are initially synthesized as large precursors called p105 and p100, are dependent on dimerization with p65, c-Rel or RelB [4,5]. In the absence of stimuli, the members of the NF-κB family form homodimers and heterodimers that are present in an inactive state in the cytoplasm bound to the κB inhibitory proteins (IκBs) (IκBα, IκBβ, IκBε, IκBγ and Bcl-3, IκBζ, the precursor proteins p100 and p105), which share multiple ankyrin repeat domains necessary for interacting with the RHD. In the established model, these proteins retain NF-κB dimers in the cytoplasm by masking the NF-κB nuclear localization sequence and the DNA-binding domain. Indeed, IκBα leads a constant shuttling of the p50/p65 complex between the nucleus and the cytoplasm [6]. In stimulated cells, IκBα is phosphorylated and degraded, thus favouring the nuclear localization of the NF-κB complex where it binds to κB sites with the consensus sequence GGGRNNYYCC (N = any base, R = purine, Y = pyrimidine) and activates the transcription of a number of target genes. Among the numerous genes regulated by NF-κB is *IκBα*. Newly synthesized IκBα can enter the nucleus, remove NF-κB from DNA and export the complex back to the cytoplasm, therefore providing a feedback mechanism to restore the original latent state. Two different intracellular pathways activate NF-κB, namely the 'classic' pathway and the 'alternative' pathway, which result in the release of NF-κB from its inhibitors and in the nuclear localization of NF-κB [7]. The canonical pathway of NF-κB activation passes through the activation of an IκB kinase (IKK) complex, composed of two catalytic subunits (IKK1/α and IKK2/β) and a regulatory subunit NF-κB essential modulator (NEMO)/IKKγ. Upon stimulation, IKK2 is involved in the phosphorylation of two N-terminal serines within the IκBs, leading to their ubiquitination and degradation through the proteasome pathway. The alternative pathway involves the processing and cleavage of the p100 precursor to p52, which is triggered by the phosphorylation of p100 by NF-κB-inducing kinase and of IKK1. In the alternative pathway, p52 mostly dimerizes with RelB in response to a limited number of stimuli such as lympotoxin B, CD40 ligand and B-cell activating factor operating in the immune system [8]. On the contrary, the canonical IKK2-dependent pathway is induced by a wide variety of stimuli. Among these are neurotransmitters such as glutamate [9], dopamine [10–12] and norepinephrine [13], as well as growth factors [14–17], beta amyloid peptide [18], oxidative stress, UV light and cytokines such as tumor necrosis factor-alfa and interleukin (IL)-1 [19] or tumour necrosis factor-like weak inducer of apoptosis (TWEAK) [20].

In brain neurons, diverse glutamate receptor subtypes, namely the ionotropic *N*-methyl-D-aspartate (NMDA) and kainate receptors and the metabotropic glutamate (mGlu) receptors, lead to NF-κB activation. Ca^{2+} signalling plays a fundamental role in NF-κB activation by ionotropic glutamate receptors [9,21]. In hippocampal neurons the opening of calcium channels is indispensable for basal NF-κB activity. Three cellular sensors of Ca^{2+} levels – calmodulin, protein kinase C (PKC) and the p21(ras)/phosphatidylinositol 3-kinase (PI3K)/Akt pathway – are simultaneously involved in NF-κB activity [22]. Stimulation of both the calmodulin kinase II and Akt kinase pathways are responsible for the upregulation of the p65 subunit of NF-κB [22]. Activation of PI3K, mitogen-activated protein kinase kinase (MEK) and PKC by mGlu5 agonists [23] or leptin [24] lead to activation of the c-Rel subunit in neuronal cells.

NF-κB in the central nervous system

In the central nervous system, NF-κB factors act as regulators of growth, differentiation and adaptive responses to extracellular signals [25–27]. The activity of NF-κB is developmentally regulated [28,29]. It has a role in adult neurogenesis [30] and in the growth of neuronal processes of maturing neurons [31–35]. Inhibiting the constitutive DNA-binding activity of NF-κB blocks differentiation and induces apoptosis. This was

reported in diverse primary neurons [29,32–34,36] and was related to the downregulation of NF-κB-mediated transcription of the B-cell lymphoma 2 (Bcl-2) family of anti-apoptotic genes [29,36]. The apoptosis of cells deprived of NF-κB activity indicates that there is a threshold of constitutive NF-κB activation below which the expression of anti-apoptotic genes and neuron survival are impaired. The presence of NF-κB in the synaptic regions has also suggested that NF-κB might be regarded as a signal transducer that transmits transient synaptic signals to the nucleus and has a role in behaviour, learning and memory formation [25,37]. By using a κB decoy DNA, the involvement of NF-κB has been shown in long-term retention of fear memory [38,39], in inhibitory avoidance memory [40] and in spatial long-term memory [41]. A forebrain neuronal conditional NF-κB-deficient mouse model confirmed the prominent role of neuronal NF-κB in memory and cognition by demonstrating that loss of neuronal NF-κB specifically impairs spatial long-term memory formation in the Morris water maze task, whereas the nonspatial working/episodic memory is unaltered [42]. p50, p65 or c-Rel factors were found to be involved in mechanisms of cognition [26,43–46]. *p50*$^{-/-}$ mice present impaired learning in an active avoidance assay [43] and show defects of short-term memory in the place recognition test [30]. They also show reduced anxiety-like behaviour in exploratory drive and anxiety tests [47]. The p65-deficient mice rescued from embryonic death on a *TNFR1*$^{-/-}$ background display spatial learning defects when challenged in a radial arm maze [44]. The *c-Rel*$^{-/-}$ mice display hypomotility and impaired hippocampal-dependent functions in contextual long-term memory and passive avoidance tasks [23,45]. The long-term memory deficit in *c-Rel*$^{-/-}$ mice correlates with defects in the long-term depression (LTD) of Schaffer-collateral synapses in the hippocampus, which is dependent on activation of the mGlu5 receptor [23]. These findings suggest that c-Rel is needed for basal synaptic transmission and maintenance of LTD in the hippocampus, whereas other members of the NF-κB family might be responsible for the induction of LTD and the late phase of long-term potentiation [42].

NF-κB complexes – bifunctional regulators of neuronal vulnerability

Besides regulating neurodevelopment and synaptic activity, NF-κB factors appear to be centrally involved in various pathological conditions associated with neurodegeneration [48–50]. These include trauma and ischemia [50–54], Alzheimer's and Parkinson's diseases [55–57] or Huntington's disease [58]. The bifunctional, neurodegenerative and neuroprotective role of NF-κB has been widely debated in the last years [59–61]. Activation of NF-κB by tumor necrosis factor protects hippocampal cells from oxidative stress [62,63], promotes neuron survival to excitotoxic noxae [64,65] and rescues cells from β-amyloid-induced apoptosis [66]. Furthermore, activation of NF-κB has a role in brain tolerance, the adaptive response induced by a subthreshold stress that preserves brain health against acute injury [67]. Among the NF-κB target genes involved in neuroprotection is the inhibitory protein, IκBα, which, by hampering aberrant NF-κB activation caused by severe ischemia or epilepsy [67], prevents brain damage. By contrast, diverse studies support the causative role of NF-κB in the degeneration of brain neuronal cells exposed to toxic stimuli. NF-κB promotes cell death in neurons exposed to excitotoxins *in vivo* [49,68] or *in vitro* [69–71], DNA damage [72], dopamine [10], mutant huntingtin [58] and β-amyloid peptide [57,73–75]. Either neurotoxic [71,76] or amiloidogenic [75] processes associated with NF-κB activation are prevented by IκBα or IKK2 inhibitors [77].

With the aim to clarify which determinants make the inducible form of NF-κB a cell death factor or a cell-survival factor in pathological conditions, recent research has shown that different NF-κB complexes are involved in the opposite regulation of neuron viability. While aberrantly activated p50/p65 dimers contribute to the apoptotic program, the c-Rel-containing dimers increase the resistance of injured neuronal cells to further damage. Studies of glutamate and IL-1β in primary neurons and hippocampal slices showed that NMDA receptor activation is associated with the rapid induction of p50 and p65 subunits from NF-κB to form the p50/p65 dimer (Fig. 1) [70,71,76]. The neuroprotection elicited by IL-1β, a cytokine involved in mechanisms of brain tolerance [78], is associated with the activation of c-Rel in addition to p50 and p65 factors (Fig. 2). Targeting p65 expression with antisense oligodeoxynucleotides prevents glutamate-mediated cell death. Targeting c-Rel, or using brain hippocampal slices from *c-Rel*$^{-/-}$ mice, abolishes the IL-1β neuroprotection without affecting glutamate toxicity [70]. In line with this evidence, the activation of NF-κB p50/p65 was found to mediate the neurotoxic effect produced by oxidative stress in HT22 immortalized hippocampal cells [79] and to contribute to both neurotoxic and amyloidogenic effects produced by the fibrillar form of β-amyloid peptide in cultured neuronal models [75]. The p50/p65 dimer is activated in the first hour of exposure to β-amyloid and precedes the expression of a characteristic pro-apoptotic gene

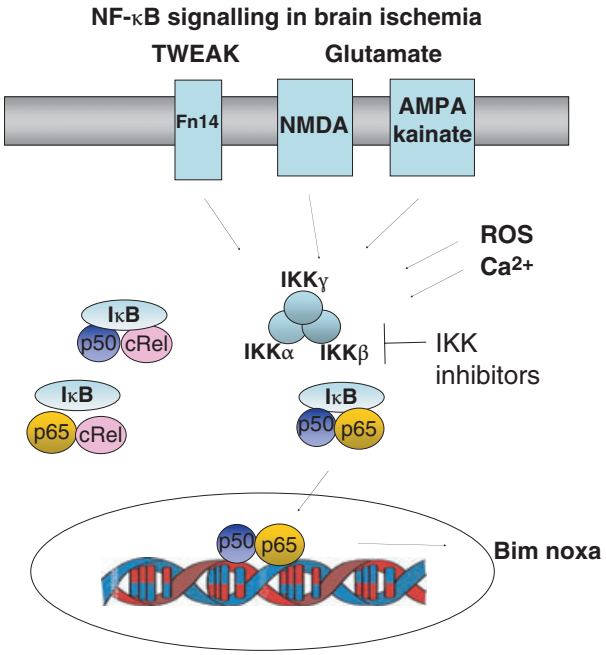

Fig. 1. NF-κB signaling in ischemia, In brain ischemia, NF-κB becomes rapidly activated in response to diverse extracellular signals (including glutamate and TWEAK) and to intracellular events [such as the generation of reactive oxygen species (ROS) and the elevation of Ca^{2+} content]. Most NF-κB activation involves the p50/p65 dimer, which induces transcription of the pro-apoptotic *Bim* and *Noxa* genes, promoting neuronal cell death. AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate.

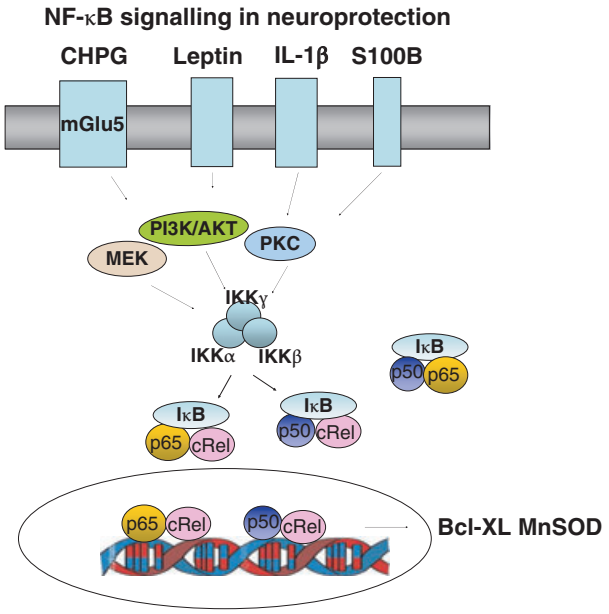

Fig. 2. NF-κB signaling in neuroprotection. Interleukin-1β, S100 calcium-binding protein B (S100B), leptin and glutamate, through the stimulation of mGlu5 receptors, activate NF-κB c-Rel dimers, but not the p50/p65 complex. These agents also activate the MEK/PI3K/PKC signaling pathways that are upstream of c-Rel dimer translocation to the nucleus. The p50/c-Rel and p65/c-Rel dimers mediate neuroprotection by inducing the expression of the anti-apoptotic genes manganese superoxide dismutase (*MnSOD*) and *Bcl-X(L)*. AKT, CHPG.

panel [75]. The relevance of c-Rel activation in neuronal cells was first outlined by evidence that the over-expression of c-Rel reproduces the anti-apoptotic response of nerve growth factor in sympathetic neurons and of insulin-like growth factor-1 in cerebellar and hippocampal cells [32,80]. Further studies showed that the neuroprotective effects produced by S100 calcium-binding protein B against NMDA toxicity in hippocampal neurons [81], or by agonists at mGlu5 receptors against β-amyloid- [82] and 1-methyl-4-phenylpyridinium toxicity [83], rely on the specific activation of c-Rel/p65 and c-Rel/p50 dimers. Targeting c-Rel factor using the RNA interference technique or *c-Rel*$^{-/-}$ neurons abolishes the expression of the anti-apoptotic genes manganese superoxide dismutase (*MnSOD*) and *Bcl-X(L)* and neuroprotection against β-amyloid toxicity by mGlu5 receptor agonists (Fig. 2) [82].

NF-κB complexes in brain ischemia

After an ischemic insult to the brain, NF-κB is rapidly activated in neurons and glial cells and, being a regulator of inflammation and apoptosis, it has been proposed to contribute to the subacute pathogenesis of the post-ischemic injury [50,51,53,84,85]. NF-κB activation and neurodegeneration results from oxidative stress and excitotoxicity and, at least in part, from the expression of TWEAK and its Fn14 receptor [20]. Using mice that express a super-repressor form of IκBα under the transcriptional control of neuron-specific enolase or glial fibrillary acidic protein, it was demonstrated that only the NF-κB loss in neurons, and not that in astrocytes, reduces the infarct size [86]. Likewise, loss of IKK2 by neuron-targeted deletion or expression of a transdominant negative mutant of IKK2 in forebrain neurons reduces ischemic brain damage in a similar way to that observed in neuron plus glia-deficient IKK2 knockout mice. Activation of IKK2 by a constitutively active transdominant mutant of IKK2 in neurons increases the infarct size [85]. It is noteworthy that neuroprotection is evident when NF-κB inhibitors lower, but do not totally abolish, NF-κB activity [85–87]. When inhibition lowers the NF-κB activity below the constitutive threshold level, the neuronal damage is exacerbated [65], in line with evidence that a critical NF-κB activity is required for cell survival and either aberrant activation or total

inhibition are detrimental [88]. To analyse which subunit of NF-κB is involved in stroke, focal cerebral ischemia was induced in mice with selective deletion of *p50* [50], *p52* or *c-Rel*, or with conditional deletion of *p65* [54]. These studies show that only mice deficient in *p50* or *p65* have a reduced infarct size when exposed to middle cerebral artery occlusion (MCAO). In order to examine the specific assembly of NF-κB subunits that form active dimers in response to ischemia in neuronal cells, together with their role in cell resistance to ischemia, primary cortical cells were exposed to oxygen-glucose deprivation (OGD), an established *in vitro* model of cerebral ischemia, and mice were subjected to permanent MCAO. It was found that the p50/p65 complex is activated in neurons during OGD as well as in ischemic brain areas of mice exposed to MCAO, whereas the c-Rel-containing dimers, c-Rel/p50 and c-Rel/p65, decrease (Fig. 1). Targeting p65 by specific small interfering RNA molecules rescues neuronal cells from the anoxic injury, whereas targeting the c-Rel factor enhances neuronal susceptibility [89]. Thus, if the p50/p65 dimer leads to cell death, the c-Rel-containing complexes drive neuroprotection. The contrasting effects played by p50/p65 and c-Rel dimers on neuronal cell survival rely on transcription of the *Bcl-2* family genes that act as major regulators of apoptosis in brain ischemia [54,90,91]. The concentrations of pro-apoptotic *Bcl-2* family members, such as the BH3-only proteins Bim and Noxa, increase during brain ischemia and are transcriptionally regulated by the p65 subunit in neuronal cells (Fig. 1) [54]. Conversely, the anti-apoptotic *Bcl-X(L)* gene is transcriptionally activated by c-Rel homodimers and heterodimers, but not by the p50/p65 complex (Fig. 2) [89]. As a demonstration of this, the content of Bcl-X(L) decreases in dying ischemic neurons and is retained in surviving cells [92–94].

The adipocyte-derived hormone leptin that strongly activates c-Rel-containing dimers such as c-Rel/p50 and c-Rel/p65, but not p50/p65, significantly reduces the infarct volume in mice exposed to permanent MCAO and rescues neurons from OGD-mediated apoptosis [24]. Both leptin-induced NF-κB activation and neuroprotection are dependent on the PI3K, MEK and PKC activity, the signalling pathways also involved in c-Rel activation and c-Rel-dependent maintenance of hippocampal LTD by agonists of mGlu5 receptors [23]. Leptin-mediated *in vitro* and *in vivo* neuroprotection is associated with expression of the *Bcl-X(L)* gene. The beneficial effect of leptin is suppressed in *c-Rel*$^{-/-}$ mice exposed to MCAO as well as in *c-Rel*$^{-/-}$ neuronal culture deprived of oxygen and glucose, confirming the pivotal role of c-Rel dimers in mediating the anti-apoptotic activity of the hormone. It is worthy of note that while neurons acutely silenced for c-Rel protein are more vulnerable to the anoxic injury, mice or cortical neurons carrying a germline deletion of c-Rel show no enhanced susceptibility to ischemia [54]; however, they become unresponsive to c-Rel-mediated neuroprotection by anti-apoptotic agents [24,71]. This suggests that c-Rel can be replaced by other NF-κB factors, during development, to guarantee the threshold of neuron vulnerability, but that it exerts a unique role in anti-apoptotic mechanisms acutely activated by neuroprotective agents to revert neurodegeneration.

Conclusions

In brain ischemia, NF-κB is involved in excitotoxic, oxidative and inflammatory events associated with neurodegeneration by displaying a dual role in the modulation of neuron survival. It has been proposed that the contrasting effects of NF-κB may depend on a different type of stimulus or target cell. According to this concept, NF-κB is neuroprotective when activated in neurons and is neurotoxic when induced in glial cells [60]. Recent progress in understanding the NF-κB dichotomy shows that within the same neuronal cell, unbalanced activation of the NF-κB p50/p65 dimer over c-Rel-containing complexes contributes to cell death secondary to the ischemic insult. While p50p/p65 promotes transcription of the pro-apoptotic Bcl-2 family members Bim and Noxa, c-Rel dimers specifically induce the *Bcl-X(L)* gene. Modification of the nuclear content of c-Rel dimers strongly affects the threshold of neuron vulnerability to anoxic injury. Drugs that by activating c-Rel-dependent transcription prevent neuronal apoptosis, such as leptin display neuroprotective activity. This latest data, by focusing on the specific role of different NF-κB components in neuronal cell survival, suggest that selective inducers of c-Rel dimers, as well as specific inhibitors of aberrantly activated p50/p65 complexes, should have much higher beneficial effects in treating ischemia than the general blockers of the NF-κB pathway tested to date.

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