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Preconditioning and neurotrophins: a model for brain adaptation to seizures, ischemia and other stressful stimuli

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Summary. The amino acid glutamate, the major excitatory neurotransmitter in the central nervous system, activates receptors coupled to calcium influx. Excessive activation of glutamate receptors in conditions such as severe epileptic seizures or stroke can kill neurons in a process called excitotoxicity. However, subtoxic levels of activation of the N-methyl-D-aspartate (NMDA) type of glutamate receptor elicit adaptive responses in neurons that enhance their ability to withstand more severe stress. A variety of stimuli induce adaptive responses to protect neurons. For example, sublethal ischemic episodes or a mild epileptic insult can protect neurons in a process referred to as tolerance. The molecular mechanisms that protect neurons by these different stressful stimuli are largely unknown but they share common features such as the transcription factor, nuclear factor kappa B (NF-κB), which is activated by ischemic and epileptic preconditioning as well as exposure to subtoxic NMDA concentrations. In this article, we describe stress-induced neuroprotective mechanisms highlighting the role of brain-derived neurotrophic factor (BDNF), a protein that plays a crucial role in neuronal survival and maintenance, neurogenesis and learning and memory.

Keywords: Preconditioning – Epilepsy – Neuroprotection – Hippocampus – BDNF – NF-κB

Introduction

Glutamate is a widespread excitatory neurotransmitter in the central nervous system (CNS) that has been shown to be involved in several important physiological processes, such as neuronal growth and differentiation during development (Lujan et al., 2005), and modulation of synaptic responses as part of learning and memory (Nicoll, 2003). However, glutamate plays an important role in the neuropathology of epilepsy (Braga et al., 2004; Morimoto et al., 2004) and ischemia by causing neurodegeneration through a process called excitotoxicity (reviewed in Novelli et al., 2005b). The N-methyl-D-aspartate (NMDA) glutamate receptor subtype plays a major role in this neuropathology (Choi, 1988). Thus, it is crucial to understand how the transition between trophic and toxic glutamatergic ionotropic receptor activation occurs, in order to develop effective therapeutic strategies. From this perspective, one important feature of glutamatergic neurotransmission is the capability to induce a significant neuronal resistance against excitotoxicity, the neuropathological process that epilepsy and ischemia share.

Epilepsy and cognition

Epilepsy is a common neurological disorder that affects approximately 50 million people worldwide (http://www.who.int/mediacentre/factsheets/fs265/en/). In adults with newly diagnosed epilepsy, impairments of memory, adaptability, attention and visual motor tasks often occur (Pulliainen et al., 2000). Cognition impairment also seems to present in patients with chronic epilepsy compared to their sibling controls (Elger et al., 2004; Roeschl-Heils et al., 2002). In addition, anti-epileptic drugs employed to treat patients with epilepsy may have a negative impact on cognition because they interfere with normal physiological processes such as learning and memory

(Sankar and Holmes, 2004). Status epilepticus is a potential complication of epilepsy that can result in hypoxic-ischemic neuronal injury in the brain. Thus, the severity of cognitive impairment may increase over the long-term disease course. Reciprocally, thrombotic stroke increases the risk for the development of epileptic seizures (Cocito et al., 1982).

Neuropathological similarities between seizures and ischemia

Focal or global ischemia induces a type of neuronal cell death similar to that seen in status epilepticus. In addition, the cellular damage is mostly restricted to neurons and not to the surrounding glia cells. The molecular and cellular events responsible for neuronal cell death in ischemia and epilepsy, although poorly understood, share several features. Transient forebrain ischemia produces cell death in hilar neurons of the dentate gyrus and pyramidal neurons in CA1 (Lindvall et al., 1992; Merlio et al., 1993; Takeda et al., 1993). Systemic administration of kainic acid at convulsant doses results in irreversible cell damage and neuron loss in the hilus of the dentate gyrus and in the CA3 and CA1 areas of the hippocampus. Saline-injected animals show no damage in the hippocampus (Gouton et al., 1998). Excitotoxic cascades caused by the overactivation of NMDA receptors leading to a massive influx of calcium are thought to be involved in such pathophysiology (Choi, 1988), although recent data suggest that the role of calcium may not be as straightforward as initially proposed (Novelli et al., 2005b). Interestingly, clinical trials using anti-excitotoxic therapies failed to show significant benefits (Lee et al., 1999). This may be related to several factors: the clinical intervention might have been outside of the therapeutic window for anti-excitotoxic treatment, antagonist concentration in the brain may not be optimal, or heterogeneity of the patient population. Finally, the antagonist administration may also prevent the glutamate-induced activation of protective pathways, such as the induction of neurotrophic factor gene expression (Marini et al., 1998).

Preconditioning and neuroprotection

Neuroprotection in vivo may be accomplished by ischemic preconditioning and cortical spreading depression. Traditionally, ischemic preconditioning requires sublethal ischemic insults via oxygen and glucose deprivation (OGD) by clamping of the carotid artery (Kirino, 2002), whereas cortical spreading depression requires the direct application of a high concentration of potassium chloride to the cortex (Chazot et al., 2002). Both methods reduce infarct size and improve functional outcome in animal stroke models. Epileptic preconditioning, a mild epileptic insult that protects neurons against status epilepticus, also protects neurons against ischemia, a phenomenon known as cross tolerance (Plamondon et al., 1999). In vitro models of preconditioning employ OGD to mimic in vivo ischemic preconditioning (Xu et al., 2002; Grabb and Choi, 1999) or chemical preconditioning where the stimulus is added in the presence of physiological concentrations of glucose and oxygen (Marini and Novelli, 1991; Marini and Paul, 1992; Marini et al., 1998).

The molecular mechanisms of preconditioned neuroprotection are still largely unknown, although NMDA receptors (Kirino, 2002), nuclear factor kappa-B (NF-κB) (Blondeau et al., 2001; Yu et al., 1999; Lipsky et al., 2001), and the extracellular signal-regulated protein kinase (ERK) pathway (Shamloo et al., 1999; Zhu et al., 2005) are all required.

Agonist-mediated stimulation of the NMDA glutamate receptor subtype, at a concentration that leads to the activation of intracellular signaling (Novelli et al., 2005a; Zhu et al., 2005) but is not at the threshold of inducing cell death in physiological concentrations of glucose and oxygen, protects vulnerable neurons against the excitotoxic effects of glutamate acting on NMDA receptors in cultured rat cerebellar granule cells (Marini and Paul, 1992). Chemical preconditioning with NMDA activates NMDA glutamate receptor subtypes to protect neurons against the excitotoxins glutamate and kainic acid in vitro and in vivo respectively (Marini and Paul, 1992; Marini et al., 1998; Lipsky et al., 2001; Ogita et al., 2003). NMDA elicits a time-dependent increase in brain-derived neurotrophic factor (BDNF) in the culture medium and TrkB tyrosine phosphorylation, suggesting that one of the mechanisms underlying the ability of NMDA to activate TrkB receptors via its intrinsic tyrosine kinase is through the increased release of BDNF, which in turn activates its own receptor in an autocrine manner. The accumulation of BDNF in the culture medium is an early event, in that it occurred within 2 min after exposure to a subtoxic concentration of NMDA (100 μM) and continued for at least 3 h. By that time, the NMDA-mediated increase in BDNF in the medium is accompanied by a concomitant accumulation of *bdnf* mRNA, suggesting that at 3 h, in addition to the release of BDNF, NMDA affects BDNF synthesis. Thus, NMDA elicits two temporally distinct responses: an early release of BDNF, and a later increase in BDNF

synthesis and release (Marini et al., 1998). Importantly, this scenario may be common to other neurons. In fact, subtoxic concentrations of NMDA and glutamate protect vulnerable cultured rat hippocampal neurons via the identical mechanism (Jiang et al., 2005).

Neuroprotective transcription factors and preconditioning

It seems clear that activation of transcription factors that activate a neuroprotective gene program must occur to induce a neuroprotective state in preconditioned neurons. One transcription factor that has been shown to play a crucial role in epileptic and ischemic preconditioning is NF-κB (Blondeau et al., 2001). NF-κB is activated by membrane depolarization as well as glutamate receptor activation (Guerrini et al., 1995; Kaltschmidt et al., 1995) and reciprocally, its activity can regulate neuronal responsiveness to glutamate (Mattson et al., 2000). NF-κB is a major survival transcription factor (Mattson et al., 2000) that is constitutively active at hippocampal synapses (Meffert et al., 2004) suggesting that NF-κB may act as a neuronal ''sensor''. Received information within the synapse is processed, leading to signal transduction pathways that result in the translocation of activated NF-κB into the nucleus where it binds to κB binding sites. Recent evidence suggests that NF-κB plays an important role in learning and memory (Meffert et al., 2004), thus providing an intriguing connection between potentiation of synaptic activity and neuroprotection. While NF-κB plays a crucial neuroprotective role in preconditioning, the downstream targets are poorly understood, but may include manganese superoxide dismutase and Bcl-2 (Mattson et al., 1997; Tamatani et al., 2000). Chemical preconditioning with NMDA increases activated NF-κB in cultured neurons and plays a crucial role in NMDA induced neuroprotection (Lipsky et al., 2001). Using a double-stranded DNA oligonucleotide prepared from the 5′ flanking region of exon 4 of the *bdnf* gene (Lipsky et al., 2001), we obtained evidence that a neuroprotective dose of NMDA (100 mg/kg IP) (Ogita et al., 2003) increases activated NF-κB in the hippocampus in a time-dependent manner in C57BL/6 mice by gel-shift assay (Fig. 1A). An increase in activated NF-κB is seen within 30 min and lasts at least up to 24 h after injection of a single dose of NMDA; a 20-fold excess of cold oligonucleotides essentially abolishes the band indicating that the band is specific. When incubated with NMDA-treated nuclear extracts, the anti-p65 antibody produced the expected gel-retardation pattern consistent with a slow migrating or

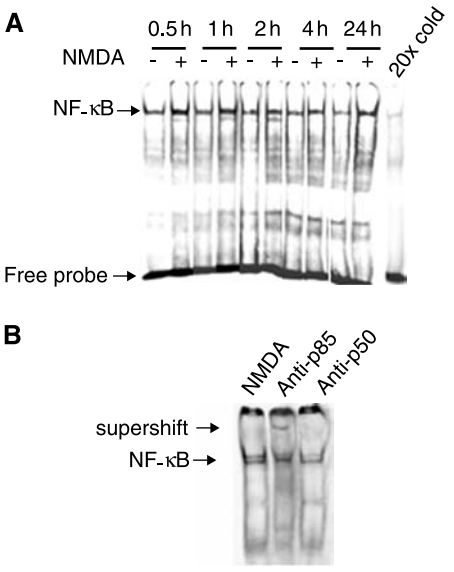

Fig. 1. A neuroprotective dose of NMDA increases activated NF-κB in murine hippocampus. Mice (C57BL/6, 30–35 gm) are subjected to a 12 h light-dark cycle at ambient temperature with free access to food and water for at least one week before use. **A** Animals are injected with NMDA (100 mg/kg, IP) prepared in PBS or PBS alone for various times (0.5, 1, 2, 4, and 24 h). At the indicated time, the animals are anesthetized, decapitated and the hippocampi from 5 animals/group removed for the preparation of nuclear extracts. The hippocampi are processed and the protein determined as described previously (Yu et al., 1999). The gel shift assay is performed using the NF-κB double-stranded oligonucleotide sequence that is based upon the 5′-flanking region of the rat *bdnf* gene (exon 4) (Lipsky et al., 2001). **B** For the supershift EMSA, a total of 5 μg/ml rabbit polyclonal antibody to NF-κB p50 or NF-κB p65 (Active Motif, Carlsbad, CA), is incubated with nuclear extracts prepared from hippocampi treated with NMDA for 2 h followed by EMSA as described (Lipsky et al., 2001)

supershifted antibody-protein-DNA complex (Fig. 1B). Curiously, anti-p50 attenuates the specific band but no supershift is observed suggesting that either the p50 antibodies are blocking antibodies or the subunit is not p50 but a related family member such as p52 (Fig. 1B). Our results suggest that a neuroprotective dose of NMDA increases activated NF-κB and that the dimer is composed of p65 and p50. Because the p65 subunit of NF-κB activates gene transcription and is activated in this model, the results suggest that NF-κB participates in the activation of the neuroprotective transcriptional program. Thus, the results obtained in the chemical preconditioning model are similar to previous results where NF-κB plays a key neuroprotective role in epileptic and ischemic preconditioning (Blondeau et al., 2001) as well as in our model of chemical preconditioning. An overview of preconditioning strategies demonstrated to protect neurons is shown in Table 1.

Table 1. Overview of preconditioning strategies

Preconditioning model	Type of stimulus	Outcome	Transcription factor and cellular targets
Epilepsy, in vivo	Mild epileptic insult	protection	NF-κB, heat shock protein, ?BDNF
Global ischemia, in vivo	Moderate ischemia	protection	NF-κB, heat shock protein, ?BDNF
NMDA, in vivo	stress	protection	NF-κB, ?BDNF
NMDA, in vitro	stress	protection	NF-κB, BDNF

NF-κB, BDNF and preconditioning

Our group discovered that one of the downstream targets of activated NF-κB is an activation of exon 4 of the *bdnf* gene. Enhanced release and synthesis of BDNF play a major role in NMDA induced neuroprotection. We have identified the activation of exon 4 of the *bdnf* gene as one of the downstream targets of activated NF-κB (Lipsky et al., 2001). To further confirm that activated NF-κB plays an important role in the expression levels of exon 4-specific BDNF mRNA levels, we performed a chromatin immunoprecipitation (ChIP). Cross-linked chromatin from hippocampal neuron cultures was sheared by sonication and subsequently incubated with antibodies specific to the p65 and p50 subunits of NF-κB and cAMP response element binding protein (CREB), which was used as a positive control, to immunoprecipitate the proteins bound to the chromatin. The immunoprecipitated DNA had cross-linked proteins removed, followed by semi-quantitative PCR using primers that amplify the region of the BDNF promoter 4 region containing the NF-κB and CRE sites. Chromatin recovered with anti-p65, anti-p50 or anti-CREB antibodies resulted in an amplification product of the predicted size (Fig. 2). These results show that a basal level of NF-κB and CREB occupy promoter 4 of the *bdnf* gene.

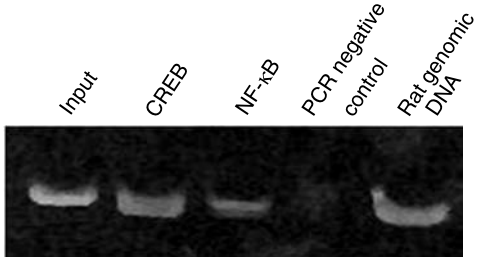

Fig. 2. NF-κB and CREB bind to promoter 4 of the *bdnf* gene. The ChIP assay is performed in cultured rat hippocampal neurons derived from embryonic day 20 fetuses as described elsewhere (Jiang et al., 2005). Proteins are crosslinked and cells are washed in ice cold PBS and recovered in PBS plus protease inhibitors for sonication as described (Orlando and Paro, 1993). Ten pulses of 30 sec with a sonicator microtip (Ultrasonic liquid processor, Misonix, 50% maximum power), separated by cooling on ice, produced fragments 500–2000 bp, an appropriate size range for this assay. After fragmenting the chromatin, the solution is incubated with a slurry of salmon sperm DNA/protein A/G agarose for 30 min to reduce non-specific binding (Takahashi et al., 2000) followed by incubation with specific polyclonal antibodies to p50 or p65 NF-κB subunits or CREB (Santa Cruz Biotechnology, Santa Cruz, CA). Immune complexes are collected using salmon sperm DNA/protein A/G agarose. After recovery of immune complexes, the cross-linked proteins are removed by incubation at 65 °C for 4 h followed by Proteinase K and RNAase digestion at 45 °C for 1 h to remove protein and RNA complexed with chromatin, followed by phenol/chloroform extraction. DNA is then recovered by ethanol precipitation along with yeast RNA as an inert carrier. The recovered DNA is amplified by PCR using specific primers from promoter 4 that flank the NF-κB or CREB binding sites, yielding products between 150–400 bp, depending on the primers used. PCR products are electrophoresed through 8 or 10% polyacrylamide gels and bands analyzed by densitometry following SYBR Gold (Molecular Probes) staining. Controls are performed in parallel with these conditions: an input DNA control for primer specificity and two references, one PCR negative control, and rat genomic DNA, a positive control

Combining these data with those reported previously by us and others, the sequence of events involved in preconditioning is shown in Fig. 3. Chemical preconditioning by NMDA receptor activation in vitro results in an influx in calcium leading to the immediate release of BDNF which in turn binds to and activates its cognate receptor, TrkB. The influx of calcium also leads to the activation of NF-κB through activation of Akt, a downstream target of the PI-3K pathway, and ERK 1/2 via the phosphorylation of its inhibitor, I-κB. Phosphorylated I-κB is ubiquinated and degraded via the 26S proteosome. Activated NF-κB translocates to the nucleus where it binds to promoter 4 of the *bdnf* gene and likely other genes such as those in the Bcl family of anti-apoptotic genes to activate transcription. Similarly, the NMDA receptor-mediated increase in intracellular calcium leads to the phosphorylation (activation) of CREB (data not shown) in the nucleus which also binds to and activates promoter 4 (Tao et al., 1998). Increased release and synthesis of BDNF results in a neuroprotective state to protect neurons against NMDA receptor-mediated excitotoxicity. Given that preconditioning models protect neurons without affecting the level and duration of the seizures (Plamondon et al., 1999) and given the diverse and crucial neuronal functions of BDNF including neuronal survival and neuroplasticity, it is possible that BDNF is one of the major neuroprotective targets of activated NF-κB. In addition, brain tolerance (preconditioning) occurs in humans (Moncayo et al., 2000; Weih et al., 1999) suggesting that our model is clinically relevant since inducing neuroprotective pathways with a preconditioner, through a physiological activation, would improve the outcome from stroke without the risk of neuronal damage associated with classical preconditioning models. Thus, it is crucial to determine the downstream

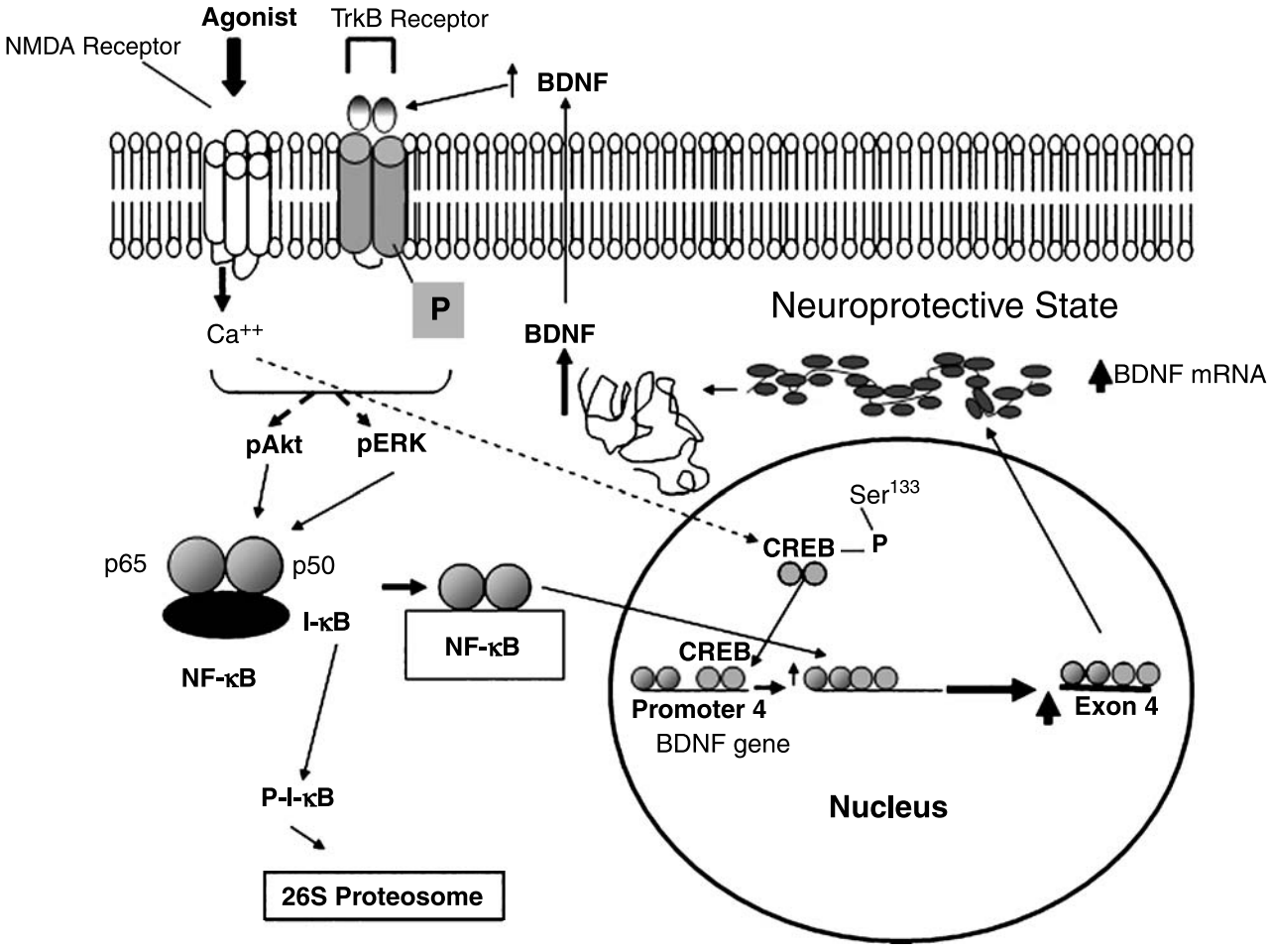

Fig. 3. Overview of NMDA neuroprotection in cultured neurons. Activation of NMDA receptors leads to the release of BDNF which in turn binds to and activates its cognate receptor, TrkB. The influx of calcium results in the activation (phosphorylation) of ERK 1/2 and Akt. Activation of various transducers activate the I-κB kinase (IKK) complex through the canonical NF-κB pathway leading to the phosphorylation and degradation of the I-κB proteins by the 26S proteosome. The active NF-κB dimer translocates to the nucleus and binds to the promoter region of NF-κB-responsive genes i.e., BDNF. CREB is activated through the phosphorylation of Ser^{133} in the nucleus and binds to promoter 4 of the *bdnf* gene and other CREB-responsive genes

neuroprotective target genes that underlie chemical preconditioning to develop innovative strategies to improve outcome from hypoxic-ischemic neuronal damage, to reduce the billions of dollars in disability costs to society annually and to provide new hope for patients with chronic epilepsy.

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