

# The Cell Permeable Peptide Strategy Is a Promising New Tool for the Prevention of Neurodegeneration

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Neurons are constantly subjected to changes in their environment and consequently they are continuously adapting to these variations. During recent years, important advances have been made in understanding the molecular events underlying the neuronal responses to extracellular signals.

The Mitogen-Activated Protein Kinases (MAPKs) are enzymes (catalytic proteins) activated by a variety of extracellular stimuli or signals, that add phosphate groups to specific amino acids within a specific protein. These enzymes form signal transduction cascades within cells. In biology, signal transduction is any process by which a cell converts one kind of signal or stimulus into another, via a sequence of biochemical reactions inside the cell. The MAPKs are the most important cytoplasmic signal transduction enzymes that are involved in many aspects of cellular regulation (Davis, 2000).

The MAPKs signaling event propagates and amplifies the external stimuli, from the plasma membrane to the cytoplasm and finally to the nucleus, with a delicately regulated mechanism, which is organized in sequential steps and involves three different key enzymes (Davis, 2000).

When signals such as the release of a neurotransmitter (a neurotransmitter is a type of molecule that carries signals between neurons) or other substances and stress

factors attain the cellular membrane they induce activation of a sequence of biochemical reactions within neurons, which are carried out by the MAPK family. In such cases the chain of steps is referred to as a "cascade" and the result is that a small stimulus elicits a large response by the phosphorylation (activation) of a series of enzymes.

In particular, the first enzyme of the cascade, MAPKKK (Mitogen Activated Protein Kinase Kinase Kinase or the first enzyme), which interacts with a second kinase, MAPKK (Mitogen Activated Protein Kinase Kinase or the second enzyme) by adding phosphate groups, to promote the transition of this inactive protein to the active form. MAPKK, in turn, will do the same to the third and the last protein kinase of the cascade, MAPK (Mitogen Activated Protein Kinase or the third enzyme) (Figure 1). The last MAPK kinase will activate (phosphorylate) many substrates including cytosolic and nuclear proteins.

Recent evidence indicates that protein-protein interactions between the kinases themselves or with substrates or other components are also a critical means of regulation (Morrison and Davis, 2003; Borsello and Bonny, 2004). It is important to emphasize once more that the signaling pathway is a sequentially ordered process (cascade of events) organized in enzymatic reactions, which are generated and controlled by many distinct protein-protein interactions.

The kinases involved in the signaling pathway interact by a protein-protein contact but can be assembled in a module by support proteins that are capable of linking several enzymes of this cascade creating multienzyme

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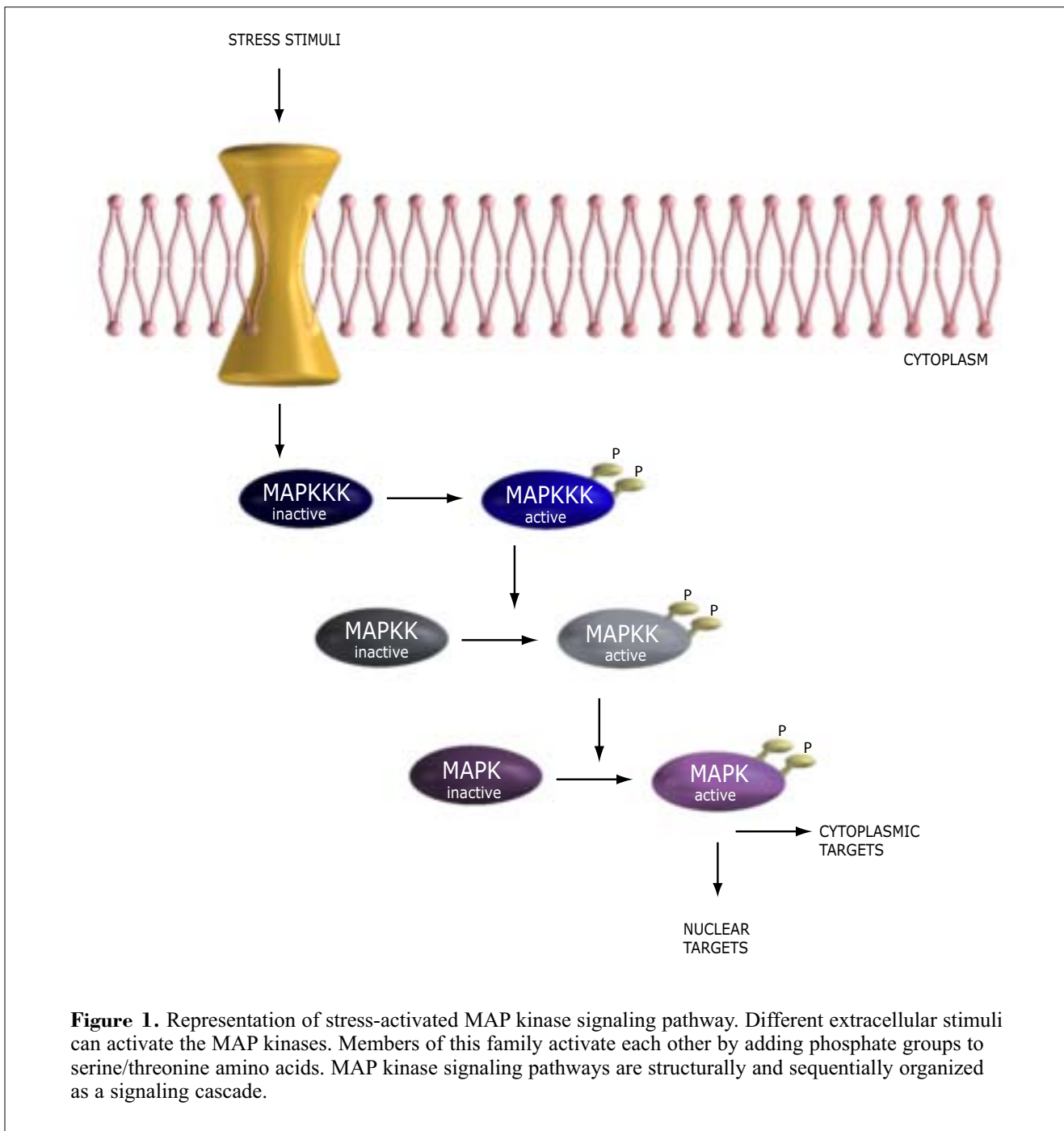
complexes (Davis, 2000) (Figure 2). This "multimerized" complex has the advantage of organizing the pathway that facilitates the interactions between proteins, accelerating the reaction and focusing the signal in a specific cellular compartment (Davis, 2000). The MAPK proteins can modulate the performances of other intracellular proteins and can induce activation of nuclear transcription factors leading to the expression of specific target genes that will eventually result in a

biological response.

There are three major groups of MAP kinases in mammalian cells:

- 1, ERK (Extracellularly Regulated protein Kinase);
- 2, p38 (p38 mitogen-activated protein kinase);
- 3, JNK (c-Jun N-terminal protein Kinase).

It has been established that the prominent role for ERK

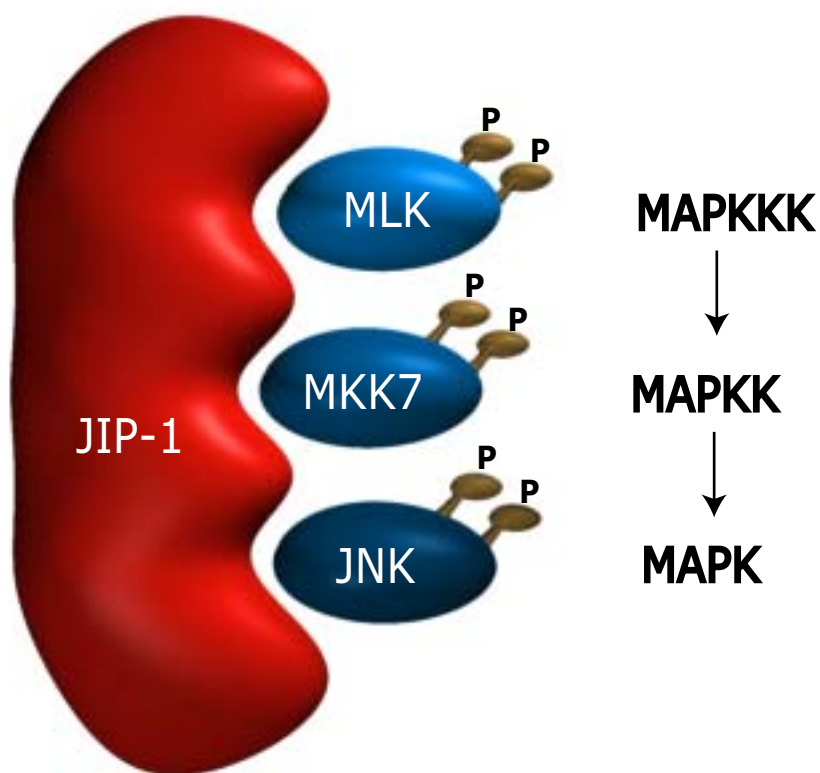


pathways is cellular differentiation, whereas p38 and JNK are mostly involved in stress responses (Gallo and Johnson, 2002). In particular JNK is implicated in response to cellular stresses such as UV,  $\gamma$  irradiation, osmotic stress, heart shock, oncogenic transformation, inflammatory responses and an excessive release of a neurotransmitter such as in excitotoxic stimulation, where excess glutamate is released (Davis, 2000).

Many of these environmental, physical and chemical kinds of insults can induce neuronal death. The mechanisms underlying the neuronal-death response involves JNK, which phosphorylates many targets culminating in the death execution program, and for this reason the JNK cascade represents a potential therapeutic target for drug development (Borsello and Bonny, 2004;

Manning and Davis, 2003). The idea is to interfere with the JNK pathway; or more precisely to inhibit JNK action (Borsello and Bonny, 2004).

Traditionally, blocking signaling along pathways is achieved through inhibition of enzymatic activities (such as preventing ATP access to the ATP-binding site of an enzyme). However, preventing the interaction between two proteins, by introducing a surplus of the specific amino acid sequence responsible for the interaction between two proteins, might also interrupt signalling cascade effects. Specific amino acid sequences can be used to target peptides to specific locations and allow for their introduction into living cells. These peptides can block key mediators of the signaling pathway, precluding protein-protein interactions.



**Figure 2.** Schematic cartoon of the JIP-1 scaffold protein. This scaffold links discrete members of the JNK pathway: MLK, MKK7 and JNK. JIP-1 seems to have the function of providing a spatial and temporal regulation of the JNK pathway and moreover, co-localizing the cascade within neurons, providing a more specific regulation of this signaling pathway after stimuli.

An example of a molecule with such an activity is the cell permeable peptide D-JNKI-1, which is able to interrupt JNK signalling pathways in living cells. This peptide was designed by Dr. C. Bonny (Bonny et al., 2001) to inhibit  $\beta$ -cell apoptosis of pancreatic islets with the main objective of treating type 1 diabetes. The generated peptide was able to block the JNK signalling, preventing induced  $\beta$ -cell death.

Since the pancreas and brain share common properties in relation to the JNK pathway as well as similar JNK-signalling components, Dr. Bonny and I (Borsello works on neurodegeneration and Bonny works on diabetes) started a collaboration for the purpose of studying key cell death mediators in order to prevent neurodegeneration and diabetes.

The D-JNKI-1 peptide is generated by linking the JNK domain (a specific region of a molecule) that is used to interact with many of its substrates called the "JNK-Binding Domain" (JBD) to the TAT48-57 transporters sequence (Vives et al., 1997). TAT48-57 is a sequence from HIV, which enters cells very rapidly and allows for the transport of JBD from the outside to the inside of neurons. JBD is able to block JNK interactions with many of its targets (Bonny et al., 2001).

It is important to emphasize that the potency and speci-

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ficity of the inhibitory action of peptide D-JNKI-1 is higher than those of the chemical inhibitors currently available for JNK, and that the peptide is very stable and effective inside cells (Borsello et al., 2003; Borsello et al., 2004).

We first tested D-JNKI-1 for preventing cell death in cortical neuronal cell cultures (cells from the cortex of the brain) that were exposed to 100  $\mu$ M of N-methyl-D-aspartate (NMDA). This substance is a glutamate agonist (which acts like glutamate itself), that induces

an excessive depolarization which can trigger the stress response in neurons. In fact this dose of NMDA would invariably induce total neuronal degeneration over the following 24 hours and provoke a rise of JNK activity to a near-maximal level within 30 minutes, followed by a decline in activity. D-JNKI-1 totally prevented the toxic effects of NMDA, and the associated activation of enzymes responsible for the execution of the cell death program (caspases) and morphological changes.

Encouraged by these results in culture, we decided to test whether the peptides would protect against neuronal death after stroke/ischemia in vivo. D-JNKI-1 will penetrate the blood-brain barrier owing to the TAT48-57 sequence, so the peptide can be administered into the Central Nervous System (CNS) in vivo through an intraperitoneal injection (injection into abdominal cavity). To investigate the potential neuroprotective effect of the peptide in vivo we blocked blood flow into the brain by middle cerebral artery occlusion (MCAO, stroke); this can be either a transient or permanent inhibition of blood flow.

We studied adult mice with transient MCAO, treated by an injection of D-JNKI-1 inside the brain. For these experiments we paid particular attention to peptide application in the post-ischemic period for its possible therapeutic application. As late as 6 hours after the insult, treatment by D-JNKI-1 reduced the lesion volume, measured 48 hours later, by more than 90%. In the past, only moderate reductions have been seen in lesion volume by neuroprotectants given 6 or even 9 hours post-lesion, by comparison the protection offered by D-JNKI-1 at 6 hours post ischemia is dramatic: at this time we observed a 93% reduction in infarcted brain tissue volume which is even greater protection than that obtained when the peptide was given before the induction of the lesion.

In the second model, permanent MCAO, in which young rats were treated with an intraperitoneal injection of D-JNKI-1, the peptide reduced the lesion by 78% at 6 hours, and by 49% as late as 12 hours post-ischemia. Protection had never before been reported at more than 9 hours post-ischemia. Moreover, protection correlated with the prevention of the increase in the activation of the specific and elective JNK target (c-Jun phosphorylation) (Borsello et al., 2003).

In the ischemia-induced brain lesion experiment, D-JNKI-1 gave a robust protection, the strongest protection ever reported in a transient model and it exhibited the longest therapeutic window reported in the permanent model. In both models, nearly equal protection was achieved at both short and long survival times (2 weeks). The longer survival time confirmed that D-JNKI-1 is not simply delaying the program of cell death but is in fact able to prevent it. Furthermore the 2-week-survival adult mice were also analyzed for the behavioral consequences of the neuroprotective treatment, using a method that measures the animal's motor performance (the rotarod). There was a clear protection of motor performance lasting throughout the 2-week period. To the best of our knowledge, behavioral protection has not been previously obtained with a neuroprotective agent given as late as 6 hours post-ischemia (Borsello et al., 2003).

D-JNKI-1 is now opening up interesting possibilities for therapeutic applications in the CNS. In fact other authors demonstrated powerful protection in different models of injury. Recently, the D-JNKI-1 protective action has been shown against hair-cell loss in animal models of sudden deafness (Wang et al., 2003) and in retinal ganglion cells following crushing of the optic nerve (Tezel et al., 2004).

Neurodegenerative diseases are an important target application for D-JNKI-1. These diseases are characterized by progressive cell loss in specific vulnerable neuronal populations of the CNS (e.g., the hippocampus in Alzheimer's and the substantia nigra in Parkinson's diseases). Although the currently available therapies afford variable improvement in disease symptoms, none of them prevents or even delays the pathological neuronal death associated with neurodegenerative diseases. The molecular basis for the underlying disease mechanisms remains to be elucidated. However, in many cases, the death of the neurons is associated with the strong activation of the JNK pathway (Bozyczko-Coyne et al., 2002). Consequently the involvement of similar biochemical events and intracellular pathways leading to cell death have been proposed in the mechanisms of Alzheimer's (AD), Parkinson's (PD) and Huntington's (HD) diseases (Bozyczko-Coyne et al., 2002).

The progressive neuronal loss, in specific vulnerable

neuronal populations of the central nervous system, is often associated with an abnormal accumulation and aggregation of proteins both inside and outside of the neurons. The basic major processes inducing neurodegeneration are considered multifactorial; one of the most important among them involves a certain protein, peculiar to each disease, and its consequential aggregation in neuronal cells. A therapeutic strategy aimed at targeting these proteins will probably be a promising one. We are currently working on two different strategies to delay the progression of chronic neurodegenerative diseases: one to inhibit the aggregation of insoluble proteins that contribute to neuronal death by targeting the protein domains responsible for aggregation, and with the other, we are developing cell permeable peptides that are able to interfere with the enzymes responsible for the production of aberrant proteins.

The promising results obtained with JNK inhibitors in acute conditions induced in animal models give us the

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hope that D-JNKI-1 could be a good molecule to test for the treatment of chronic diseases. However such diseases are characterized by a progressive cell loss that develops over many years and it is not difficult to predict that prolonged and systemic administrations of D-JNKI-1 will probably generate unacceptable side effects. For this reason the D-JNKI-1 application will need further investigation and toxicological studies.

The cell permeable peptides are a valuable tool that offer the possibility to perturb intracellular signaling pathways and this strategy opens up new and promising opportunities to prevent neuronal death.

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