



Use of cell-permeable peptides to prevent neuronal degeneration

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The loss of neurons is a hallmark of neurodegenerative disorders and evidence suggests that this occurs through an apoptotic mechanism. Following an insult, neuronal cells activate signal transduction pathways that lead to cell death and the establishment of the pathological state. The mechanisms underlying the cell-death response involve protein kinases, which phosphorylate many substrates and culminate in changes in gene expression. Traditionally, attempts at blocking such signaling targeted the phosphorylation of the substrates. However, preventing the interaction between two proteins using specific peptides might block the function of key mediators in signaling cascades. A cell-permeable peptide designed specifically to inhibit c-Jun N-terminal kinase action proved successful in *in vivo* models of neuronal degeneration following ischemia. Here, the recent findings that highlight the potential of this approach for therapeutic application are reviewed.

A signal transduction cascade is defined by the biochemical events that are mobilized by a cell to convey and interpret a given signal, such as ligand binding to specific receptors, as well as the consequent output from the cell. Accumulating evidence has indicated that the recruitment of the c-Jun N-terminal kinase (JNK) signaling pathway has an important role in the development of different neuronal pathologies. As do all signal-transduction processes, the mobilization of the JNK cascade involves both the activation of modifying enzymes, such as kinases, and the recruitment of non-enzymatic components, including adaptors and scaffold proteins. Until recently, small cell-permeable chemicals aimed at blocking the propagation of the signaling cascades that occur within cells were almost exclusively aimed at blocking enzymatic activities. The recent emergence of peptidic transporters that can deliver linked molecules to the cytoplasm of cells now enables the interruption of signaling cascades (and, therefore, the manipulation of the cell-response) by blocking the interaction between two proteins along the signaling pathway without directly interfering with the enzymatic activities.

The JNK signaling cascade

Cells respond to extracellular stimuli through the activation of signal transduction pathways, which induce modifications of pre-existing proteins and culminate in changes in gene expression. A crucial component of the signal-transduction process is the activation of protein kinases, which coordinate diverse cellular activities, including mitosis, programmed cell death, motility and metabolism [1]. The mitogen-activated protein kinases (MAPKs) are a family of enzymes that can modulate the activities of other intracellular proteins by adding phosphate groups to serine or threonine amino acid residues, which occur in the well-defined consensus sequence P-X-S/T-P. Prior to the activation of the MAPKs, similar phosphorylation events on other enzymes generate self-propagating cascades. This sequential process enables one kinase in the cascade to phosphorylate and thereby activate its downstream substrates, and enables MAPK cascades to function as highly sensitive amplifiers, converting graded low-level inputs into ultrasensitive outputs. Using this mechanism, the cascade can effectively filter out noise and respond properly to stimuli. These activating kinases work through direct protein-protein interactions. However, the components of the signaling pathways might also be assembled by scaffold or adaptor proteins that create multienzyme complexes [2]. Examples of scaffold proteins include the JNK-interacting proteins (JIPs), which aggregate upstream activating elements of the JNK cascade [3]. JNK is a member of the MAPK family (Figure 1).

The JNK pathway is implicated in multiple physiological processes but it is more responsive to stress stimuli, such as UV radiation, X-rays, heat shock, neurotrophic factor withdrawal and inflammatory cytokines. Because of its ability to be recruited by a diverse array of stimuli and to control the transactivating potential of the transcription factor c-Jun, the JNK signaling pathway is thought to be implicated in many pathological conditions, including cancer, stroke, heart disease, inflammatory pathologies, diabetes and neurodegenerative disorders. Studies using genetically modified mice have provided strong evidence for a role of JNK in programmed cell death. For example, *Jnk1*- and *Jnk2*-deficient fibroblasts from mouse embryos fail to undergo apoptosis in response to classical apoptotic stimuli [4], and neurons from mice in which the *Jnk3* gene is disrupted were partially resistant to excitotoxic

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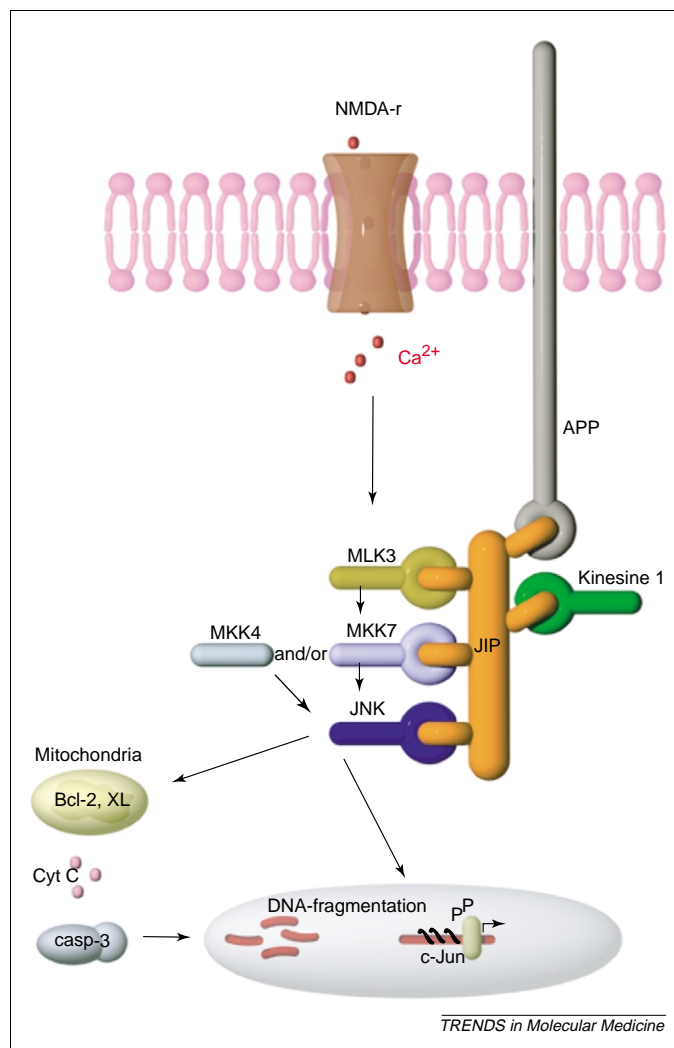


Figure 1. The JNK signaling pathway in neurons. Following NMDA stimulation, opening of the NMDA receptor (NMDA-r) leads to calcium influx (Ca^{2+}) that activates the JNK signaling pathway. The cascade proceeds through the sequential interaction of mitogen-activated protein kinases (MAPKs). The first kinase (MLK3) interacts with the second, MKK4 and/or MKK7, which in turn triggers the activation of JNK. Activated JNK might phosphorylate several different substrates, including nuclear factors (c-Jun, ATF2, E1k), receptors (glucocorticoid receptor), surface membrane proteins (APP) or mitochondrial factors (Bcl-2, Bcl-xL). Loss of mitochondrial transmembrane potential is a common feature of apoptosis, triggering the release of Cyt C, which, in turn, activates the caspase-3. The JIP-1/IB1 scaffold protein can further structurally organize the MAPK signaling module. The JIP-1/IB1 protein links MLK3, MKK7 and JNK, thus providing a temporal and spatial regulation of the JNK pathway. Recent studies demonstrate that JIP-1/IB1 also binds to APP and kinesine-1.

neuronal death [5]. These data provided evidence that important cellular responses can occur through the interaction of small signaling modules [6].

Because JNK might be partially inactive in many cell types in the normal state, its signaling pathway is a potential therapeutic target for the prevention of cell death that is caused by a variety of stimuli. Therefore, it was originally suspected that blocking JNK activity might only target stressed cells and would only impair detrimental responses without affecting the function of normal cells. Furthermore, because activation of JNK is the final step in the cascade before the signal enters the nucleus (through the activation of c-Jun), it was believed that targeting JNK instead of upstream events (such as receptor binding)

might enable a lengthening of the therapeutic window (the time following an insult after which a therapeutic intervention might still prove beneficial). This could have important consequences in conditions such as brain ischemia, in which patients usually reach medical centers 6–10 h after the ischemic event.

JNK function in the brain

JNK expression in the brain is ontogenetically regulated [5,7]. Thus, JNK1 is widely present in the prenatal brain and its expression is rapidly downregulated after birth. By contrast, the distribution of JNK3 mRNA does not substantially change between prenatal and postnatal brains [7]. The different distribution of JNK mRNAs in the nervous system and neural cell lines suggests both the existence of different functions for the distinct JNK isoforms, as well as compensatory redundancy. In the human central nervous system (CNS), all three JNKs are present but JNK3 α 1 and JNK1 α 1 are the two most abundantly expressed forms [8]. Furthermore, in the brain, the three JNK isoforms are predominantly present in an activated form [5,9–11]; under normal basal conditions, basal JNK activity is 15–30-fold higher than in other tissues [12,13]. JNK can be further activated by the exposure of neurons to stresses [13]. This high basal activity of JNK in the brain suggests that JNK also has a physiological role in the nervous system, such as contributing to the modulation of axonal growth or promoting repair (sprouting). Pathological events (degeneration and cell death) require the further activation of JNK (especially JNK3) in these tissues (Figure 1).

Disease states of the CNS: JNK-mediated excitotoxicity

Excitotoxicity might contribute to the pathogenesis of neuronal death that is induced by insults such as hypoxia–ischemia [14], epilepsy [15], trauma [16] and neurodegenerative diseases [17]. The crucial role of JNK3 in excitotoxicity was demonstrated in *Jnk3*-knockout mice, because the hippocampal neurons in these animals were protected against excitotoxic cell death [5]. Furthermore, mice with an inactive form of the major JNK target, c-Jun, were resistant to excitotoxic neuronal cell death [18]. However, *Jnk1*^{-/-} and *Jnk2*^{-/-} mice are not resistant to excitotoxic cell death, indicating that different JNK isoforms can modulate diverse responses and that the activation of JNK3 is required in excitotoxicity [19]. Excitotoxic events, as well as the activation of inflammatory cytokines and the loss of trophic factors, generate ischemic neuronal cell death. In transient ischemic models, degenerating neurons exhibit the activation of JNK and c-Jun phosphorylation in the peri-infarcted region [20,21]. In permanent ischemic models, increased JNK activity is detected within the ischemic lesion [14]. This is indirect evidence that suggests an involvement of JNK in the cell-death process. One recent study addressed the role of JNK in the pathology more directly: using a gerbil model of transient ischemia, it was demonstrated that intracerebral administration of SB203580, a MAPK inhibitor, reduced ischemic cell death [22]. However, caution in interpreting these data is necessary as a result

of the low specificity of the inhibitor used, as well as the high dosage required to cross the blood–brain barrier.

Neurodegenerative diseases

Similar biochemical events and intracellular pathways leading to cell death have been proposed to be involved in Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD). Events such as excitatory toxicity, oxidative stress, mitochondrial defects, inflammatory reactions and abnormal protein folding and aggregation have all been postulated to have varying roles in the pathogenesis of these diseases (Figure 2). Furthermore, emerging evidence suggests an involvement for JNK in the excitotoxic cell-death processes that occur in different neurodegenerative states [23]. Therefore, potential treatments might be based on the inhibition of the JNK death pathway. However, neurodegenerative diseases are progressive illnesses that develop over several years. For this reason, promising therapies should be started early in the time course of the disease. Additionally, the neuroprotective agent should be administered for a long period of time to prevent neuronal loss. However, because the prolonged and systemic administration of JNK blockers will probably generate unacceptable side

effects, therapeutic strategies aimed at limiting the biological activities of these molecules in the affected tissues will be required for the treatment of chronic diseases (for example, by administering the peptide by local injection, such as in the *Substantia Nigra* for PD).

D-JNKI-1: a cell-permeable and protease-resistant peptide to selectively interrupt JNK signaling

The most frequently used strategy to prevent kinase activities along the JNK pathway is to block signaling. However, preventing the interaction between two proteins further downstream might also interrupt signaling cascades. This could be achieved by the introduction of a molar excess of the peptides sequences that mediate the interaction between the protein partners [24–26]. Intracellular delivery is achieved by linking these sequences to special peptidic transporters, including TAT_{48–57}, antennapedia and others [27]. Because of its short length (10 amino acids) and its good efficacy in crossing the cell membranes of numerous different cell types, the TAT_{48–57} sequence from HIV appears to be the most popular method for the intracellular delivery of peptides [28]. TAT has been linked to various sequences, including BH4-domain peptides from Bcl-xL, NEMO-binding-domain peptides (NBDs, which block NF- κ B signaling), TRAF peptides, peptides derived from the signaling protein PSD95 (TAT-NR2B9c), cyclin D–Cdk4/6 derived peptides, hypoxia-inducible factor-1 (HIF-1) peptides and many others [25,29–33]. In each of these cases, TAT appears to transport sufficient amounts of the blocking peptides into cells to achieve complete inhibition of the relevant protein–protein interactions (in the range 1 μ M–1 mM). TAT has also been linked to larger proteins, such as Bcl-xL, for use as a delivery system to reduce ischemic brain injury [34–36].

Similar peptides have been engineered to block the interaction between JNK and its target, c-Jun, and have been used in animal models of neuropathological disorders [23]. JNK interacts with most of its substrates through a ~15 amino acid sequence called the JNK-binding domain (JBD). One such peptide from the JIP-1/IB1 protein, which appears to be the most stable interacting partner of JNK, has been fused to TAT_{48–57} to generate the JNKI (JNK-inhibitor) peptide [26]. A similar peptide, which uses the JBD from the c-Jun protein has also been studied [37]. These inhibitors completely inhibit the phosphorylation of c-Jun by JNK and block the apoptotic response to different stress conditions.

The *D-retro-inverso* form of JNKI, which was called D-JNKI-1 (it is identical to JNK1, but is composed exclusively of D- instead of L-amino acids and is synthesized in a reverse order to preserve functionality), has also been synthesized. This peptide mimetic enables the circumvention of several problems traditionally associated with peptides or proteins, such as proteolytic instability. This is an issue that is particularly relevant to neuronal cells and basic peptides (such as TAT_{48–57}), which are recognized by the neuronal proteases involved in peptide processing [38]. Interestingly, D-JNKI-1, despite an ~15-fold reduction in its ability to prevent the JNK/c-Jun interaction when compared with JNKI,

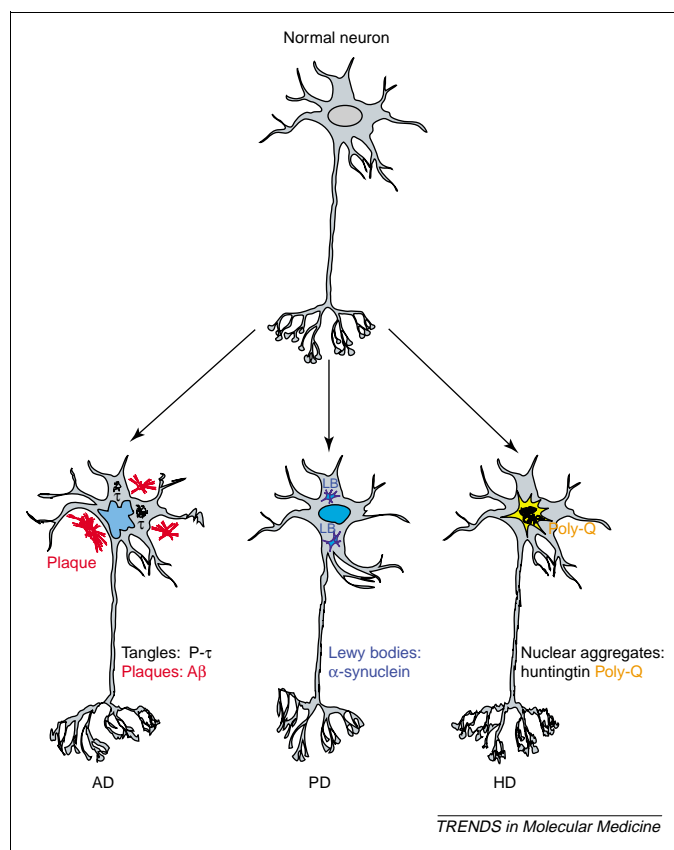


Figure 2. Schematic representations of neuronal degeneration. Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) share common features, such as neuronal dysfunction, synapse damage and mechanisms involving death pathways. These disorders are characterized by progressive neuronal loss and by deposits of abnormal proteins in the brain, in the form of aggregates or plaques. In particular, AD exhibits the characteristic extracellular amyloid plaques (A β) and intracellular tangles (P- τ), whereas in PD, the dying dopaminergic neurons exhibit aggregated proteins (α -synuclein) in the form of cytoplasmic Lewy bodies (LB). In HD, mutations in the huntingtin protein (Poly-Q) generate toxic aggregates in the cell nuclei that are associated with cell death.

can still completely prevent the phosphorylation of c-Jun inside cells and protect against apoptosis.

A general property of the JNKI peptides is their extremely potent action inside cells, well above what might be expected from *in vitro* biochemical data. For example, using a classical *in vitro* JNK kinase assay, the 50% maximal inhibitory concentration of JNKI, with c-Jun as substrate, is $\sim 1 \mu\text{M}$ [26,37]. Total inhibition is essentially achieved at $10 \mu\text{M}$. In cells, $1 \mu\text{M}$ is sufficient to completely block the phosphorylation of c-Jun, and 1nM is sufficient to decrease the apoptosis of pancreatic β -cells induced by IL-1 β by 50%. This is probably because the amount of intracellular c-Jun is extremely low, thus reducing the amount of peptide required for inhibition. It should be noted that the conventional kinase inhibitors, such as SB203580 or SP600125, which block the binding of ATP [39,40] are measured by a different method: the 50% inhibitory concentration is usually measured at a fixed concentration of $10 \mu\text{M}$ ATP, which is significantly below the usual intracellular concentration of ATP. As a result, higher concentrations of the conventional inhibitors are required, which results in the adverse consequence of lowering specificity through the inhibition of other kinases.

D-JNKI-1 prevents ischemic damage with an exceptional therapeutic window

The D-JNKI-1 cell-penetrating peptide causes the total prevention of excitotoxicity *in vitro* and a powerful (>90%) neuroprotection in two ischemic models *in vivo* [20]. Remarkably, this innovative treatment is still effective in protecting the brains of adult mice and young rats when administered 6–12 h after the onset of ischemia. In these models, the negative effects of ischemia on motor

behavioral functions were totally prevented and there were no major side effects of D-JNKI-1 at dosages 10-fold above the minimal dosages required for maximal protection [20]. These data indicate that neuronal cell death might still be prevented 6–12 h after the onset of stroke and that JNK itself might be a realistic target for therapeutic intervention. Obviously, exhaustive toxicological studies will be required before enabling the clinical evaluation of D-JNKI-1. Such toxicological studies have not yet been performed for the use of the TAT_{48–57} transporter peptide.

Strategies to delay the progression of chronic neurodegenerative diseases

The blocking of JNK to slow the progression of neurodegenerative diseases (AD, PD and HD) might lead to unacceptable side effects. However, a conceptually similar therapeutic strategy could be used. For example, novel peptides might be designed that will inhibit another common feature of neurodegenerative diseases, the aggregation of insoluble proteins that contribute to neuronal death (Figure 3) [41]. It might be possible to engineer peptides to prevent the formation of α -synuclein aggregates in PD. Similarly, toxic aggregates of the mutated huntingtin protein (which form through interactions of the polyglutamate tail) in HD could be targeted. Finally, considering the strong association between the amyloid β -chain ($\text{A}\beta$) and AD, therapeutic strategies aimed at lowering the concentration of $\text{A}\beta$ in the brain might prove beneficial for the treatment of the disease. Two proteases process the amyloid precursor protein (APP) to give $\text{A}\beta$: a first cleavage with α -secretase generates a soluble and a non-amyloidogenic fragment, whereas cleavage with β -secretase results in the production of $\text{A}\beta$. Because β -secretase is rate limiting for

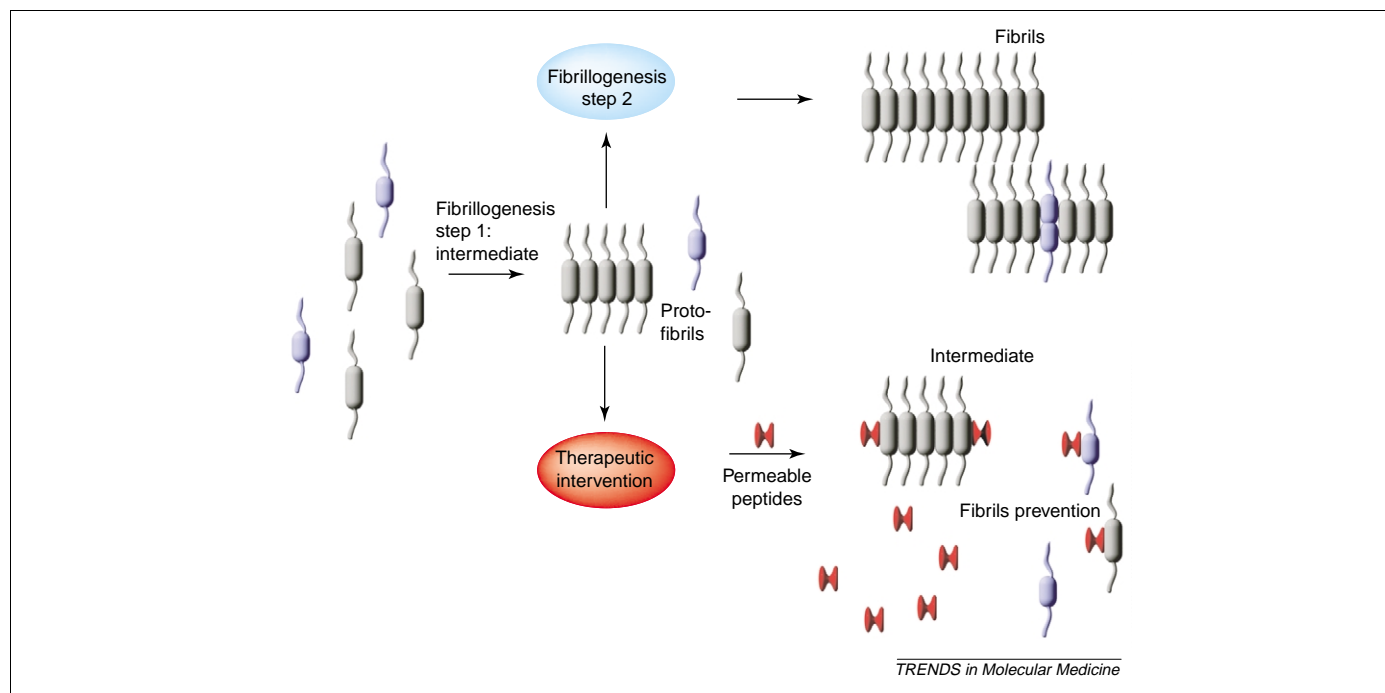


Figure 3. Protein aggregation in neurodegenerative diseases. Protein aggregation of misfolded proteins appears to be a key component of neurodegeneration. The first pathological step in the pathology (step 1) is the formation of misfolded intermediate proto-fibrils of the native protein, which, in a second step (step 2) of aggregation, become stabilized by the formation of an oligomeric sheet structure that gives rise to an insoluble fibril conformation. Cell-permeable peptides (red) that can interact with the protein domain responsible for toxic fibrillogenesis might inhibit the accumulation of aberrant proteins (fibrils) and, therefore, prevent neuronal cell death.

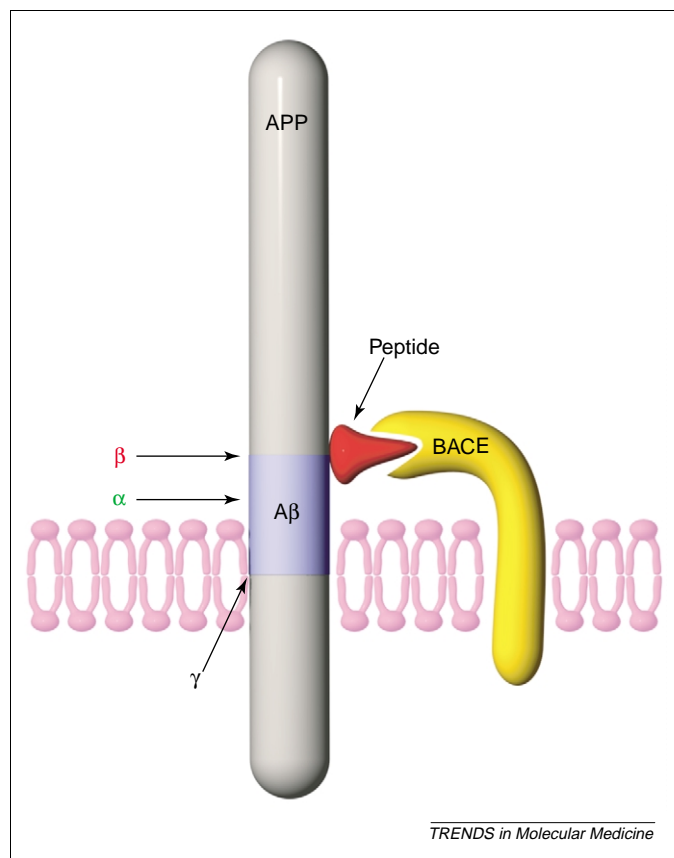


Figure 4. Modulation of amyloid-precursor protein processing by cell-permeable peptides. The proteolytic processing of the amyloid- β -protein precursor (APP) has a pivotal role in the development of Alzheimer's disease. Cleavage of the amyloid- β protein precursor might occur via two pathways, both of which involve the action of proteases called secretases. One pathway, involving β - and γ -secretases, liberates amyloid- β protein (A β), which is a protein that is associated with neurodegeneration. The alternative pathway, involving α - and γ -secretases, precludes A β formation. Inhibition of aberrant A β production should prevent, or at least limit the extent of, AD pathophysiology. Cell-permeable peptides that target the β -cleavage site on APP might offer a new therapeutic approach. BACE (in yellow) is β -secretase.

the production of A β , the APP site processed by this enzyme is probably a relevant target for designing a cell-permeable peptide to specifically interfere with amyloidogenic cleavage (Figure 4).

Cell-penetrating peptides: limitations and practical uses

Although it has been established that cell-penetrating peptides might be used in small-animal studies to manipulate signaling pathways, an obvious limitation for human therapy is the cost of such molecules. For example, the doses of D-JNKI-1 used in animals correspond to an amount of peptide close to 1 g for the treatment of a human [20]. This indicates that two possibilities are available for realistic therapeutic intervention. First, a cell-penetrating peptide should be active in cell-culture assays (as opposed to pure *in vitro* studies) at concentrations below 1 μ M. This would enable concentrations to remain below the 100- μ M limit at which most peptide transporters show signs of toxicity [42]. Few cell-penetrating peptides function at these concentrations, including D-JNKI-1 and TAT-NR2B9c [20,33]. Others peptides, such as NBD, are significantly beyond this range (with an effective concentration of >200 μ M). Second, it

will probably be wise to restrict the use of these peptides to small organs, including the eyes and ears, or to small organ transplants such as pancreatic islets. This is of particular interest because TAT-linked molecules do not leak from cells, suggesting that they stay at the site of injection [37]. The long biological activity of D-JNKI-1 (and other D peptides), for example, might be useful for the treatment of chronic diseases that are typically associated with these organs (e.g. glaucoma or presbycusis). It might also be envisaged that linking the highly stable D-TAT transporter to other chemicals could increase their resident time inside organs. By taking advantage of the localized effect of TAT-linked peptides, it might also be possible to use high dosages, which would be toxic following systemic administration.

Concluding remarks

Neuroprotective strategies aimed at blocking excitotoxic neuronal cell death might improve brain recovery following the type of insults that occur in ischemia or neurodegenerative disorders. Cell-permeable peptides that target protein-protein interfaces and, in particular, those that block the JNK-c-Jun interaction, such as D-JNKI, have been shown in animal models to be potent inhibitors of neuronal cell death. It will be important to clarify to what extent the exceptional properties of the D-JNKI peptide, in terms of its specificity [34], localized effects [43], long residency within the cell and long-lasting biological activities [25], are involved in the protection observed *in vivo* [20,43]. Careful side-by-side comparison with classical inhibitors will be required. Nevertheless, proof of principle has now been established, demonstrating the powerful capacities of cell-permeable peptides to modulate biological responses in the brain.

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