protocols were generated with the pClamp software package (Axon Instruments, Foster City, CA) running on an IBM AT computer. Single-channel currents were recorded with an Axopatch 1C patch-clamp amplifier (Axon Instruments), digitally sampled at 2500 Hz, low-pass–filtered at 1 kHz (Bessel filter, −3 dB), and written to computer disk. Analysis of single-channel data was accomplished with in-house software. We corrected raw current traces for leakage and capacitative current by subtracting an average of current activated in the absence of intracellular Ca^{2+}. We idealized single-channel events with a half-amplitude threshold detection routine. Solutions were applied locally to a membrane patch with a glass pipette containing seven separate perfusion lines. Flow in each line was driven by gravity and regulated by computer-controlled solenoid valves. Solution exchange time was approximately 100 ms. Experiments were done at 21° to 24°C.

29. As in Fig. 4A, an apparent initial decrease in opening probability was routinely observed after trypsin application. One interpretation of this observation is that the putative BK channel–inactivating gate, once cleaved from the rest of the channel protein by trypsin, has a higher affinity for its binding site than does the attached gate. Thus, for channels that are exposed to trypsin while inactivated, the cleaved inactivating gate will dissociate from its receptor site more slowly, giving rise to a temporary decrease in P_o.

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A Strategy for Delivering Peptides into the Central Nervous System by Sequential Metabolism

Nicholas Bodor,* Laszlo Prokai, Wei-Mei Wu, Hassan Farag, Sastry Jonalagadda, Masanori Kawamura, James Simpkins

Most peptides do not enter the central nervous system because of their hydrophilic character and the presence of peptidolytic enzymes in the lipoidal blood-brain barrier. To achieve brain delivery of a peptide conjugate, an opioid peptide (enkephalin) was placed in a molecular environment that disguises its peptide nature and provides biolabile, lipophilic functions to penetrate the blood-brain barrier by passive transport. The strategy also incorporates a 1,4-dihydrotrigonellinate targetor that undergoes an enzymatically mediated oxidation to a hydrophilic, membrane-impermeable trigonellinate salt. The polar targetor-peptide conjugate that is trapped behind the lipoidal blood-brain barrier is deposited in the central nervous system. Analgesia was observed with "packaged" enkephalin but not with the unmodified peptide or lipophilic peptide precursors.

The blood-brain barrier (BBB) is the major obstacle for the development of centrally active peptides. The capillaries in the brain parenchyma possess high-resistance, tight junctions between the endothelial cells (*1*). The cells also lack pores; thus, the brain capillary endothelium behaves like a continuous lipid bilayer. Diffusion through this layer, the physical BBB, is largely dependent on the lipid solubility of the solute. Water-soluble molecules (for example, glucose, essential amino acids, and glutamate) enter the brain almost exclusively by carrier-mediated transport (*2*). Most peptides, such as the naturally occurring enkephalins, are hydrophilic and do not cross the BBB, because of the absence of specific transport systems in the membrane. Their metabolic instability also implies that the highly active neuropeptide-degrading enzymes (*3*), such as the capillary-bound aminopeptidase (*4*), arylamidase (*5*), and enkephalinase (*6*), constitute an enzymatic BBB for peptides that results in their rapid cleavage.

Various strategies have been applied to direct centrally active peptides into the brain. An invasive procedure that includes surgical implantation of an intraventricular catheter followed by pharmaceutical infusion into the ventricular compartment delivers a metabolically unstable peptide only to the surface of the brain (*7*). Transient opening of the tight junctions by the intracarotid infusion of an osmotically active substance (mannitol, arabinose) in high concentrations (>1 M) may facilitate an indiscriminate delivery of molecules that otherwise cannot cross the BBB (*8*). However, this procedure is accompanied by severe toxic effects, which can lead to inflammation, encephalitis, and seizures. These invasive procedures are only justified for some life-threatening conditions and are not acceptable for less serious illnesses.

A noninvasive method for peptide delivery into the central nervous system (CNS) has been suggested that uses the formation of chimeric peptides (*9*). This strategy relies on the presence of specific receptor-mediated transcytosis systems in the BBB for certain larger peptides such as insulin, insulin-like growth factor, transferrin, and albumin. Covalently coupling (for example, through disulfide bonds) a nontransportable peptide to these transport vectors results in a chimeric peptide that can also undergo receptor-mediated transcytosis, and the active peptide can be released by its enzymatic cleavage in the CNS. However, these carriers are not brain-specific, as uptake by nonneural cells or cells outside the CNS has also been shown (*10*). Low amounts of the peptide relative to the carrier molecule and the receptor-based cellular transport mechanism that has physiologically limited transporter capacity (saturable) also prevent pharmacologically significant amounts from entering the brain. Finally, release of the active peptide from the conjugate has not been documented.

Our approach is an enzyme-based strategy and is distinct from a simple pharmacologically based approach in which peptide "prodrugs" are applied that are lipophilic esters or amides of the molecule (*11*). Although the acquired lipophilicity of these prodrugs may assure penetration to the BBB (and to other membranes), this is not the sole factor involved in the transport of a peptide into the CNS. BBB transport of cyclosporin, which is one of the most lipid-soluble peptides, is paradoxically low as a result of peptide degradation (*12*). The enzymatic BBB is also circumvented by our approach. The prototype of the system (Fig. 1) has been designed and evaluated for the enkephalin analogs, (D-Ala^2)-Leu-enkephalin and (D-Ala^2)-(D-Leu^5)-enkephalin. Both the COOH-terminus and the NH_2- terminus of the molecule have been modified in such a way as to increase the lipid solubility of the peptide and also to prevent cleavage by the BBB aminopeptidases. Additionally, the 1,4-dihydrotrigonellinate redox targetor (T) exploits the unique architecture of the BBB, which allows for the influx of the lipid soluble neutral form, but it is not permeable to the positively charged form. The redox targetor has proved to be widely applicable for brain targeting of a variety of substances (*13*), and its attachment alone results in brain-specific delivery for small molecules such as dopamine (*14*).

The enkephalins are sensitive to cleavage by endopeptidases at the Gly^3-Phe^4 peptide bond. Cholesteryl, a bulky and lipophilic steroidal moiety (L), provides an ester function that increases the lipid solubility and also hinders the COOH-terminal portion of the peptide from being recognized by peptide-degrading enzymes.

Center for Drug Discovery, College of Pharmacy, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32610.

*To whom correspondence should be addressed.

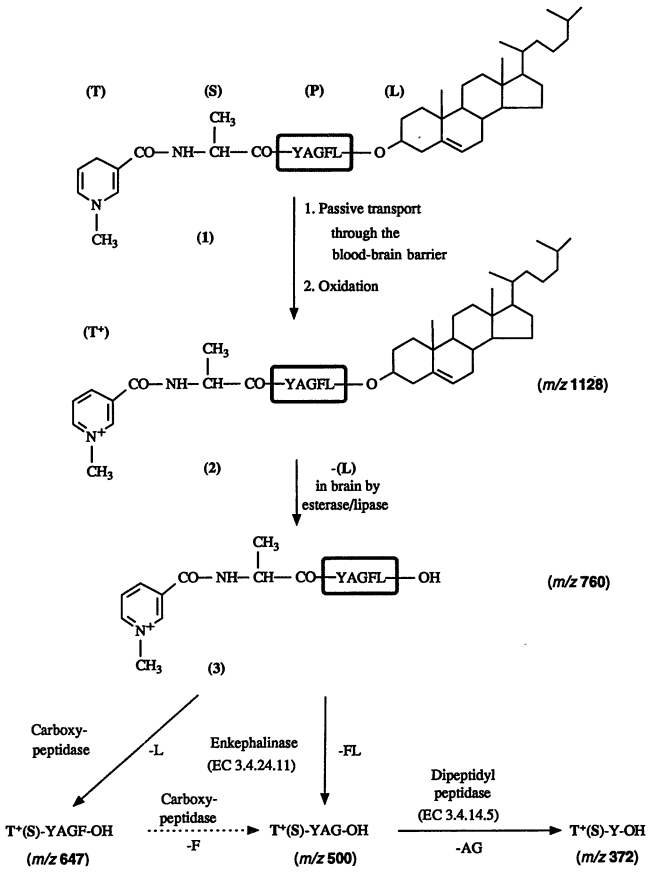

Fig. 1. Brain delivery of peptides by sequential metabolism. The "packaged" molecule (compound 1) with its peptide nature disguised enters the CNS by passive transport without proteolytic degradation because of its high lipophilicity. The targetor (T) function is converted by enzymatic oxidation [$NAD^+ \leftrightarrow$ NADH coenzyme (nicotinamide adenine dinucleotide is the reduced form of NAD^+)] to compound 2 possessing a membrane-impermeable ionic group (T^+), the biolabile lipophilic protection is cleaved by esterase or lipase, and the relatively stable, locked-in targetor-peptide conjugate (compound 3) may interact with specific receptors and be processed by peptidases. The protected molecules in the scheme were obtained by a solution-phase, sequential deprotection-coupling procedure (*t*-butoxycarbonyl chemistry) starting with the leucine cholesterate and finally coupling (DCC, dicyclohexyl-carbodiimide) with nicotinic acid. We obtained compound 2 by the subsequent quaternization with dimethyl sulfate, and its reduction with sodium dithionite yielded compound 1. As an analytical standard and the subject of in vitro evaluation, we obtained compound 3 by solid-phase peptide synthesis. The resin-bound peptide (AYAGFL; Ala-Tyr-Ala-Gly-Phe-Leu) was coupled with nicotinic acid, then quaternized (dimethyl sulfate), cleaved from the support by hydrogen fluoride, and purified by preparative reversed-phase liquid chromatography. The compounds prepared during this study have been fully characterized by chromatography and mass spectrometry (fast atom bombardment and electrospray ionization).

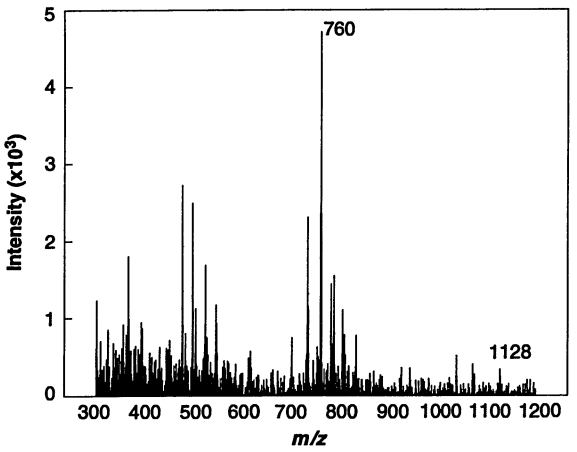

Fig. 2. Detection of the peptide conjugates in the brain tissue by electrospray ionization mass spectrometry after systemic administration of compound 1. Sprague-Dawley rats weighing 200 to 300 g were used as experimental models. The delivery system (compound 1), dissolved in a vehicle consisting of ethanol and 50% w/w hydroxypropyl-β-cyclodextrin (1:1) vehicle, was injected intravenously through the tail vein at a dose of 20 mg per kilogram of body weight. After killing the animals by decapitation, we collected the brain tissue and homogenized it in cold 1 M acetic acid. After centrifugation for 15 min at 12,500*g*, the supernatant was removed and was passed through Supelclean LC-18 cartridges. The poorly retained compounds were eluted with 3% (v/v) acetic acid, and we collected the sample by eluting with 70% methanol plus 30% water containing 3% (v/v) acetic acid. The solvent was removed under dry N_2 stream, and the reconstituted (in 50% methanol plus 50% water containing 3% acetic acid) sample was analyzed by electrospray ionization mass spectrometry at a flow rate of 5 μl/min. In the sample collected 15 min after intravenous administration of compound 1, (D-Ala²)-Leu-enkephalin, compound 2 (*m/z*, 1128) can be detected, and compound 3 (*m/z* 760) is present in an estimated 500 to 700 pmol per gram of tissue level. Quantification was based on a comparison of the peak intensity to that obtained from the brain sample of an untreated animal spiked with a known amount of compound 3. In tissue collected 1, 2, and 4 hours after systemic administration, compound 2 can no longer be identified, and the quantity of compound 3 is proportionally (with approximately 40- to 60-min half-life) decreased with time.

This part of the molecule is, however, labile toward esterase or lipase, which permits its removal after delivery. The lipases or esterases expose the peptide unit that can interact with specific receptors or that may serve as a substrate for various neuropeptide processing and degrading enzymes. A spacer function (S) is also incorporated to preserve the integrity of the peptide unit by spatially separating the important segment of the molecule (YAGFL; Tyr-Ala-Gly-Phe-Leu) from the targetor (T). This spacer may be another amino acid residue or residues. The selection of an L-alanyl spacer can be justified on the basis of the suggested involvement of alanyl aminopeptidase in the enkephalinergic transmission in the CNS (*15*). The peptide unit in our delivery system appears as a perturbation on the bulky molecule dominated by the lipophilic steroidal portion and the targetor, which also prevents recognition by the peptidases.

We evaluated our approach with electrospray ionization mass spectrometry, which

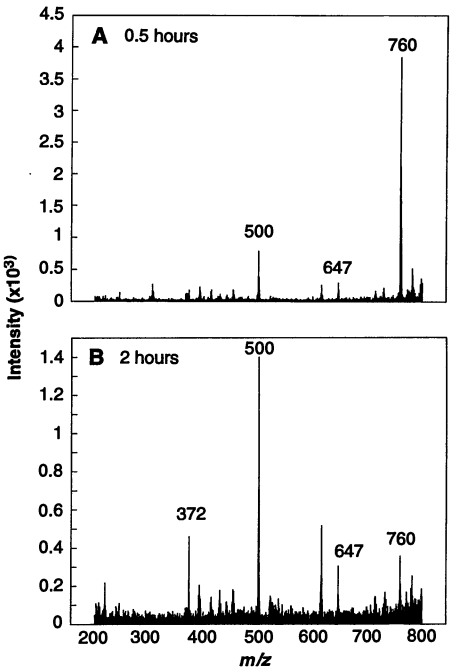

Fig. 3. Processing of the brain-delivered peptide conjugate in vitro. The targetor-peptide conjugate (compound 3) is processed by the brain peptidases similarly to the unmanipulated compound, (D-Ala²)-Leu-enkephalin. Compound 3 (30 nmol) was added to 1 ml of rat brain homogenate (20%, w/w, in pH 7.4 tris buffer), and the mixture was incubated at 37°C. Aliquots (250 μl) were removed (**A**) 0.5 and (**B**) 2 hours after incubation, and electrospray ionization mass spectra were obtained after sample preparation identical to that described in the legend to Fig. 2. Proteolytic cleavages are identified by the *m/z* of the products, and the corresponding structures are shown in Fig. 1.

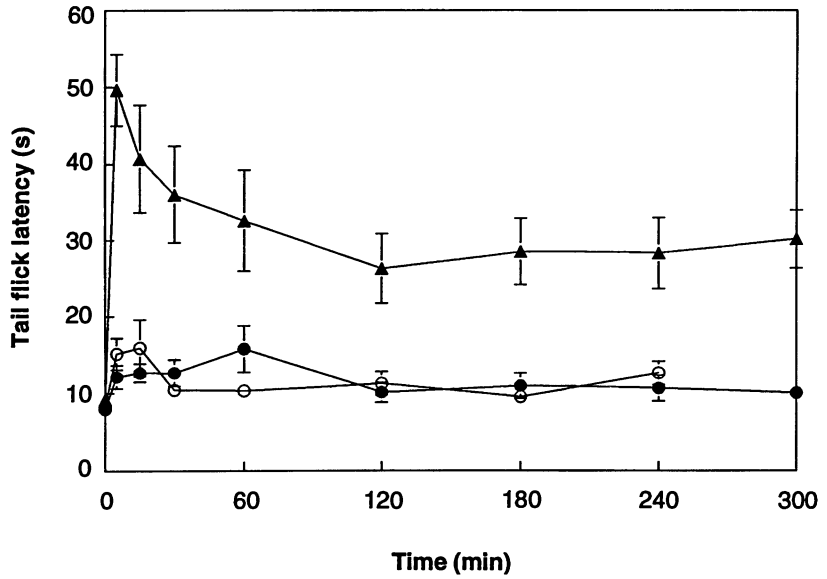

Fig. 4. Central nervous system effect induced by the peptide delivery system. Injection of the modified peptide produces analgesia, but the intravenously injected unmanipulated peptide lacks central activity. Sprague-Dawley rats weighing 200 to 250 g were intravenously administered equimolar doses (8.8 μmol per kilogram of body weight) of compound 1, or the parent peptide (D-Ala^2)-(D-Leu^5)-enkephalin in a vehicle (1:2:1 mixture of dimethyl sulfoxide, ethanol, and 50% aqueous 2-hydroxypropyl-β-cyclodextrin solution, respectively). Tail flick latency, a measure of the spinal cord–mediated analgesia, was determined before administration (control), then at 5, 15, and 30 min, and 1, 2, 3, 4, 5, and 6 hours after the intravenous administration of the compounds. The time between the presentation of a focused beam of light and the reflexive removal of the tail from the stimulus was recorded. Ten animals were used for each compound. A sustained and statistically significant increase in the tail flick response was obtained for the enzyme-based, brain-delivery system (▲), whereas the parent peptide (●) showed no effect compared with the group of animals injected with the vehicle solution (○).

provides the specificity necessary to monitor the biotransformation processes that occur after delivery. In vivo distribution studies with rats as experimental models have shown that on systemic administration the modified peptide can partition by passive, nonsaturable transport into the brain, which is inaccessible to the unmanipulated compound. At this first step, the modified peptide enters the brain because of its lipoidal nature. The targetor moiety, however, undergoes an enzyme-mediated oxidation analogous to the endogenous NAD(P)H ↔ NAD(P)$^+$ coenzyme {NAD(P)H is the reduced form of nicotinamide adenine dinucleotide phosphate [NAD(P)$^+$]} associated with numerous oxidoreductases and cellular respiration (*16*). This redox reaction converts the dihydrotrigonellinate to the hydrophilic, membrane-impermeable trigonellinate ion; thus, it remains trapped behind the BBB, as shown by the presence of a mass-to-charge ratio (m/z) of 1128 (compound 2) in the electrospray ionization mass spectrum of the brain extract (Fig. 2). Oxidation of compound 1 (Fig. 1) in the periphery, on the other hand, results in its rapid secretion from the body, because the pyridinium salt, compound 2 (Fig. 1), is easily eliminated by the kidney and bile. Consequently, no detectable amount of the trigonellinates was found in the blood samples collected 5 to 10 min after systemic administration.

The removal of the cholesteryl (L) by esterase or lipase occurs subsequent to or simultaneously with the brain-targeting enzymatic oxidation. The targetor-peptide conjugate compound 3 (Fig. 1) occurs at 500 to 700 pmol per gram of brain tissue 15 min after the intravenous administration of the modified peptide. However, a half-life of about 40 to 60 min was observed for the conjugate of (D-Ala^2)-Leu-enkephalin, which indicates its processing or degradation by peptidases. The fate of the targetor-peptide conjugate compound 3 has been investigated with an appropriate in vitro experiment (Fig. 3). As with the (D-Ala^2)-Leu-enkephalin analog, the neutral endopeptidase or enkephalinase (*17*) is most probably the major degrading enzyme for the peptide conjugate, and the action of other peptidases (carboxypeptidase, dipeptidyl peptidase) can also be reasonably assigned (*3*). The conjugate of the (D-Ala^2)-(D-Leu^5)-enkephalin exhibits increased resistance to the carboxypeptidases and to the enkephalinase.

We observed a significant and prolonged increase in the latency of the tail-flick response, a measure of the central analgesic activity, of the experimental animals after the intravenous injection of the modified peptide (Fig. 4). The targetor-peptide conjugates (compound 3) are weak opioids. The 50% inhibitory concentration (IC_{50}) measured with [^{3}H]diprenorphine-based competitive assay is about 10^{-7} M—an order of magnitude less than the IC_{50} of about 10^{-8} M for the enkephalin analogs. At the measured concentration of 500 to 700 pmol per gram of tissue, even these weak opioids exert significant analgesic activity. The enkephalin analog may also be released slowly from the targetor-peptide conjugate in vivo and contribute to the CNS activity. No analgesia was observed after the intravenous administration of the unmanipulated enkephalins, which are membrane-impermeable and unprotected toward various peptide-degrading enzymes, or after the injection of the partially conjugated (either with the targetor or with cholesteryl) peptides. Modification of the spacer (S) function may allow for the peptidolytic release of the parent peptide from the conjugate at a desired rate, depending on the therapeutic objective.

In conclusion, our method, which is based on a chemical delivery system, can be used for the brain-delivery of peptides. When the obstacle represented by the BBB is overcome, the promise of biologically active peptides to become a future generation of high-efficiency neuropharmaceuticals may be realized.

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