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Biousian glycopeptides penetrate the blood–brain barrier

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Abstract—Clinicians have long desired the ability to introduce either exogenous or endogenous neuropeptides directly into the brain in order to alter brain chemistry, but have been thwarted by the blood–brain barrier (BBB). The BBB blocks the introduction of most peptides and proteins into the brain. Glycosylation can be employed as an effective and practical strategy that allows the systemic use of neuropeptides in vivo. A series of glycopeptides based on the Leu-enkephalin analogue YtGFS*-$CONH_2$ led to greatly enhanced stability in vivo and effective penetration of the BBB. Transport through the BBB hinges on the biousian nature of the glycopeptides. That is, the amphipathic glycopeptides possess two conflicting solubility states; one state that is completely water soluble, and another at water-membrane phase boundaries. Multiple lines of evidence suggest that the BBB transport is absorptive endocytosis. Several Leu-enkephalin analogues studied showed antinociceptive potencies greater than morphine. Moreover, these δ-selective glycopeptides lacked many of the μ-opioid side effects generally associated with classical opiate analgesics. The biousian design was extended to much larger glycopeptides (16–17 residues) related to β-endorphin, which also penetrated the BBB and produced antinociception in mice. Plasmon-waveguide resonance (PWR) studies showed that the amphipathic helices bound to membrane bilayers with micromolar to low nanomolar K_D's. The presence of diverse endogenous neuropeptide transmitters and neuromodulators in the human brain is potentially applicable to the treatment of a wide range of behavioral disorders.

1. Introduction

The discovery of a plethora of endogenous neuropeptide transmitters suggests the potential for treatment of disease states with a vast pharmacopoeia based on endogenous neuropeptides from the brain itself.[1] It was once believed that neuropeptides would replace many older drugs, and be free of many side effects typically associated with small molecule drugs. This goal has remained elusive due to the blood–brain barrier (BBB) that limits the entry of exogenously applied peptides into the brain from circulation. Peptide neurotransmitters and neuromodulators in the CNS play many firmly established roles in health and disease, and new roles continue to be discovered at a prodigious rate. Practical methods for the transport of neuropeptides into the brain could make this new pharmacopoeia a real possibility.

We now report the use of glycosylation as a design element to promote the transport of pharmacologically active neuropeptides analogues into the brain. Using the well-characterized enkephalins and their endogenous opioid receptors as a model, we have synthesized a series of O-linked enkephalin glycosides **2**–**6**, including the unglycosylated parent, **1** (Table 1). Studies in mice and rats show that glycopeptide **5** produces potent and long-lasting antinociceptive activity superior to that of morphine. Using a multidisciplinary approach we have examined the molecular conformation of glycosylated enkephalins in solution, in the presence of membranes, and with membrane models; binding and agonism at the μ, δ, and κ opioid receptors; transport through the BBB; analgesia and side effects in vivo. The findings suggest that glycosylation can be employed as a strategy to allow the systemic use of neuropeptides allowing for the

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Table 1. Opioid activity of glycosylated enkephalin analogues

YdGFLS*-$CONH_2$ glycoside	Glucoside moiety	δ Binding (nM)	μ Binding (nM)	MVD IC_{50} (nM)	GPI IC_{50} (nM)
1	H (peptide control)	2.1	7.5	2.7	25
2	β-D-Glc	2.4	7.6	1.6	34
3	α-D-Glc-(1→4)-β-D-Glc	9.9	30.8	1.7	52.6
4	$[\alpha$-D-Glc-(1→4)$]_2$-β-D-Glc	3.8	15	7.7	71.7
5	β-D-Gal-(1→4)-β-D-Glc	17.3	40	5.72	34.8
6	α-D-Gal-(1→6)-β-D-Glc	5.6	36.6	6.06	43.8

development of novel approaches to the treatment of disease with minimal side effects.

A series of glycopeptides were synthesized[2] with varying types of O-linked glycosides attached to Ser[6] (Fig. 1). O-Linked glycosylation of the relatively lipophilic Leu-enkephalin C-terminal amide YdGFS*-$CONH_2$ led to enhanced surfactant properties[3] of the molecule, which in turn led to increased interaction with membranes and membrane mimics. Although these relatively short glycosylated neuropeptides had no defined conformation in aqueous solution (e.g., they existed as random coils), in the presence of micelles or membranes they adopted a very restricted and well-defined set of conformations, as indicated by CD and ^1H NMR analysis.[4]

The peptide 'message' chosen for these studies is a selective δ-opioid agonist, but also has appreciable agonist effects at the μ-opioid receptor, which is believed to be essential for maximal analgesic efficacy. No appreciable κ-opioid binding has been observed. It has been previously shown that the glycosylation site (S*) must be at the C-terminal 'address' of the enkephalin in order to avoid interfering with binding to the opioid receptors.[5] Interestingly, increased glycosylation caused a modest reduction in affinity for the μ-opioid receptor, but had virtually no effect on δ binding or efficacy, antinociception in this case.[6] This is consistent with Schwyzer's membrane compartment theory,[7] which suggests that the μ-receptor binding site resides in a more hydrophilic environment than the binding site for the δ-opioid receptor.

2. Results

Classical pharmacological theories of BBB transport suggest that peptides are not lipophilic enough to diffuse into the brain.[8] Glycosylation decreases lipophilicity even further, and transport would not be predicted, based on the 'Rule of Five.'[9] Despite this cautionary note, modestly δ-selective glycopeptide Leu-enkephalin analogues were synthesized using solid-phase Fmoc methods (Fig. 2) and tested for BBB transport activity. Increased stability and greatly increased transport rates in rat brain were observed for the glycosylated enkephalins (Fig. 3). Previous studies with the glucoside **2** indicated that the increased transport was due to a saturable mechanism, thus further ruling out simple diffusion. Reversible interaction of the glycopeptides with the membrane is believed to promote transport through the brain capillaries by transcytosis.[10] Several other possible modes of transport (simple diffusion and receptor-mediated processes) have been ruled out.[11] Maximum transport rates (and maximum biological effects) are observed when the optimum degree of glycosylation is achieved. For this peptide, the disaccharide produces both the optimal transport and stability in vivo. In general, glycosylation leads to enhanced stability of the peptide 'message' in both serum and brain. The identity of

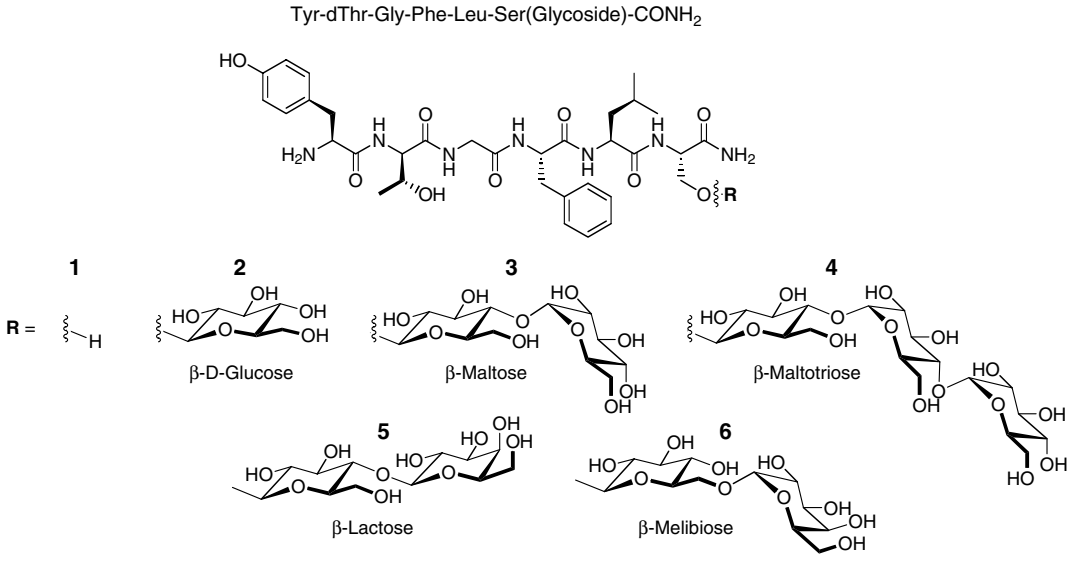

Figure 1. Glycosylated enkephalin analogues.

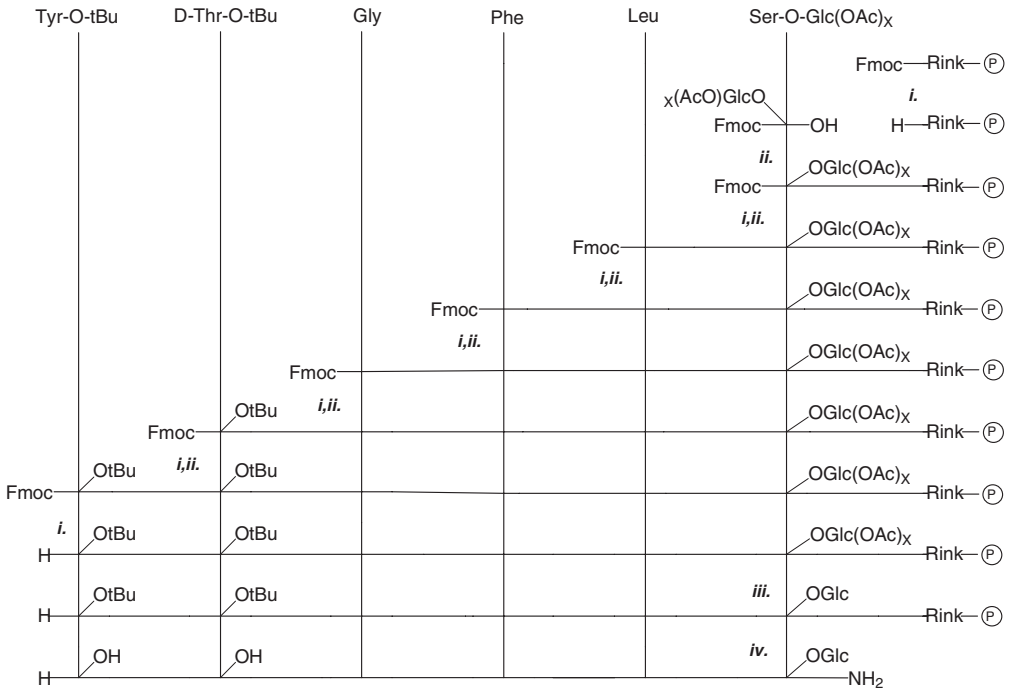

Figure 2. Solid-phase glycopeptide synthesis. Glycopeptide **2** is illustrated: i. 25% $C_5H_{10}NH$/DMF, DMF wash; ii. BOP/HOBt/$EtNiPr_2$; iii. $H_2NNH_2{\cdot}H_2O$; iv. wash with cleavage cocktail, $F_3CCOOH/Et_3SiH/H_2O$/PhOMe/CH_2Cl_2 (9:0.5:0.5:0.05:1).

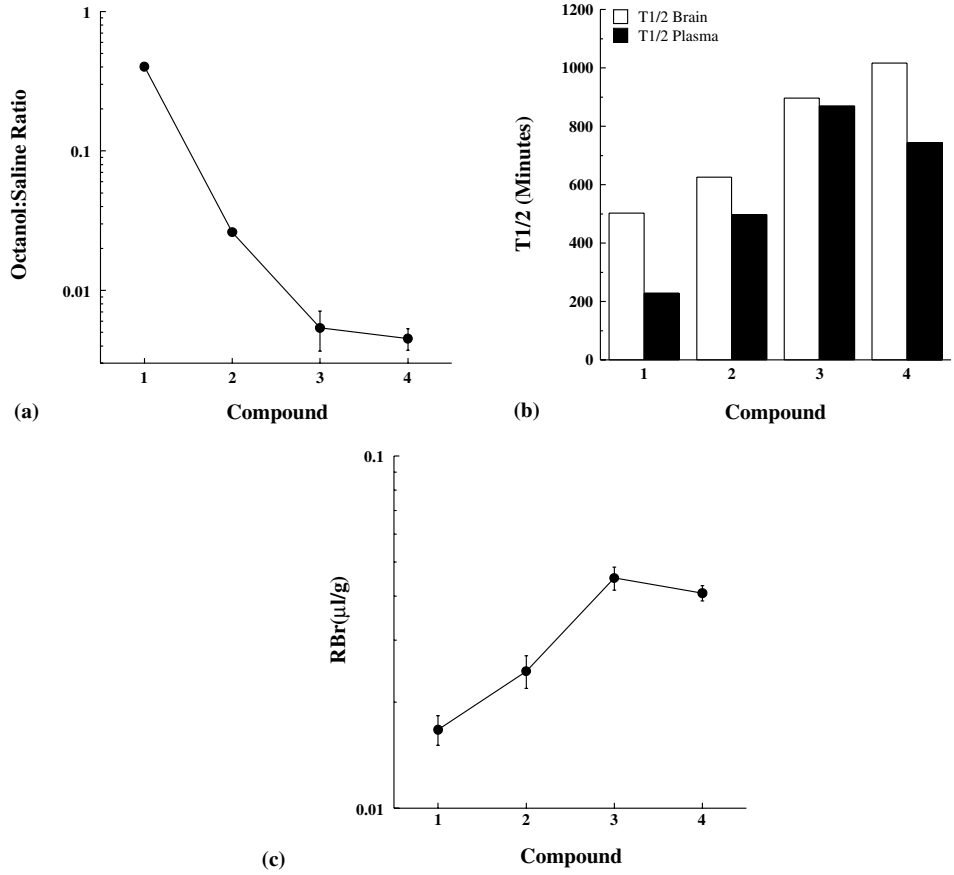

Figure 3. Glycopeptide stability and transport. (a) Octanol–saline distribution for the unglycosylated peptide **1** and glycopeptides **2**, **3**, and **4**. The addition of 1, 2, or 3 glucose units to the opioid peptide message significantly decreases lipophilicity. (b) The in vitro stabilities of the peptide and glycopeptides were measured in mouse brain and serum. Increased glycosylation led to significant increases in stability in both brain and serum. Brain stability increased with each additional glucose. However, in the serum, the stability of the trisaccharide was lower than that of the disaccharide. (c) Brain delivery of the peptides measured by in situ perfusion studies. Addition of glucose to the peptide significantly increased uptake. Uptake to the brain was improved further for the disaccharide, giving the maximal delivery. The trisaccharide produced no further increase in uptake.

the individual sugars does, however, contribute to the overall biological effect, which is a product of both BBB transport rates and the stability of the peptide in serum.

The extent of antinociception was shown to be comparable to, or even superior to the effects of morphine in mice after intracerebroventricular injection (icv) and intravenous injection (iv) administration[12] using the warm water tail flick assay.[13] The representative glycopeptides all produced full agonist effects in these assays with the potencies exceeding that of morphine on a μmol/kg basis in some cases (Fig. 4). Additional analgesic assays involving visceral, chemical, and inflammatory pain states were also used to gauge the effectiveness of **2** and **5**, after iv and subcutaneous injection (sc) administration of the drugs.

Two well-known effects of morphine in rodents increase in locomotor activity[14,15] with stereotypic patterns of movement,[16] and increases in muscular rigidity, including Straub tail.[17] Unlike morphine and other μ-selective opioids, at equivalent sc A_{90} antinociceptive doses, or even supramaximal doses, the glycopeptide analgesics produced minimal increases in locomotor activity, and did not produce Straub tail (Fig. 5). These results were confirmed in two different strains of out-bred mice.

3. Discussion

Operating on the hypothesis that the biousian nature[18] of the glycopeptides facilitates penetration of the BBB, further studies were performed with much larger helical amphipathic peptide sequences. Helices are the most commonly occurring secondary structural elements in globular proteins, accounting for one-third of all the residues.[19] Linus Pauling first proposed the α-helix as an important motif of secondary structure in proteins in 1948,[20] interestingly, without any experimental evidence.[21,22] Segrest theorized the amphipathic helix (a.k.a. amphiphilic helix) to be an important structural motif of integral membrane proteins in 1974.[23] It is estimated that over 50% of all α-helices in nature are amphi-

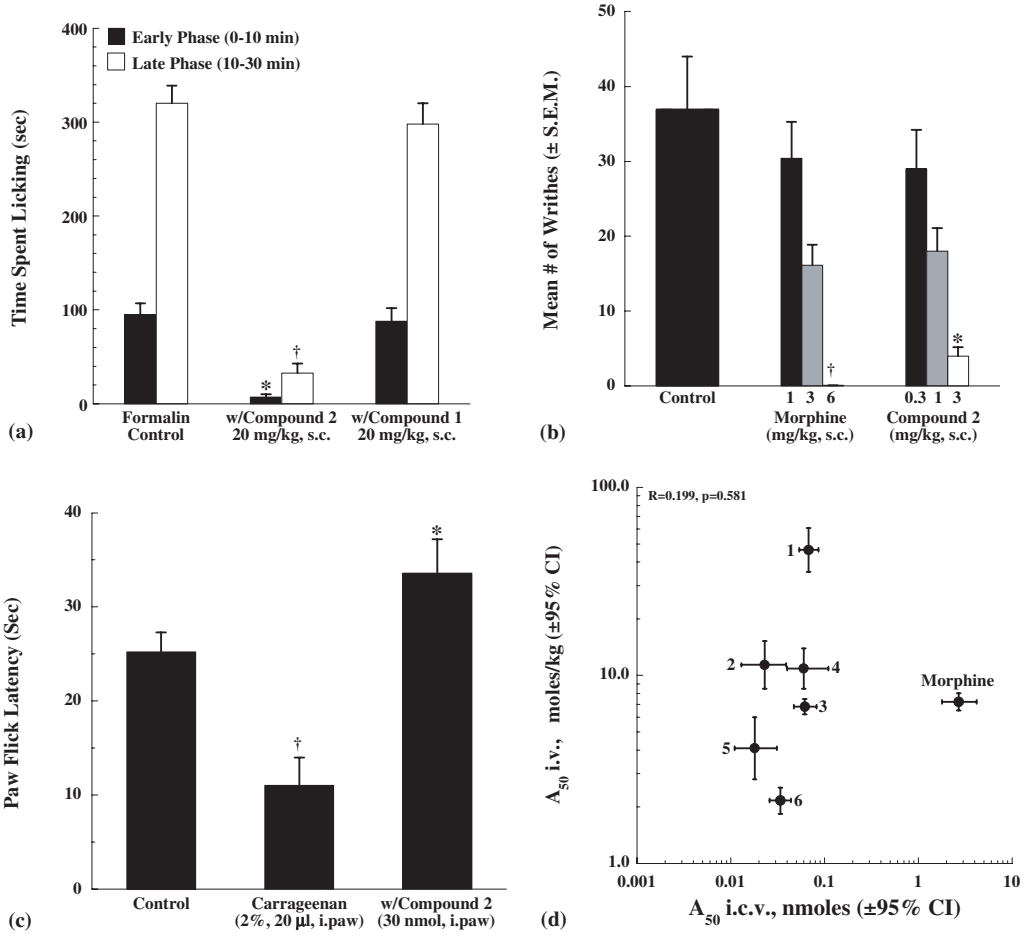

Figure 4. Analgesic (antinociceptive) effects. The glycosylated enkephalins showed strong analgesic activity in tests of antinociception after peripheral administration, which are more clinically relevant than the tail flick assay. (a) Mouse formalin paw test, glycopeptide **2**, sc; (b) mouse abdominal constriction test, glycopeptide **2**, sc; (c) mouse paw inflammation test with carrageenan, glycopeptide **2**, i.paw; injection of **2** into the contralateral injection had no antinociceptive effects; (d) antinociceptive effects (mouse tail flick) of glycosylated enkephalins (A_{50} values) after icv administration (*X* axis), and after iv administration (*Y* axis). Morphine has been included as a reference point, but has been excluded from the correlation values, shown on the upper left part of the diagram. The observed analgesia after iv injection correlates most strongly with glycopeptide stability (Fig. 3b), and brain transport values (Fig. 3c), rather than the icv potency.

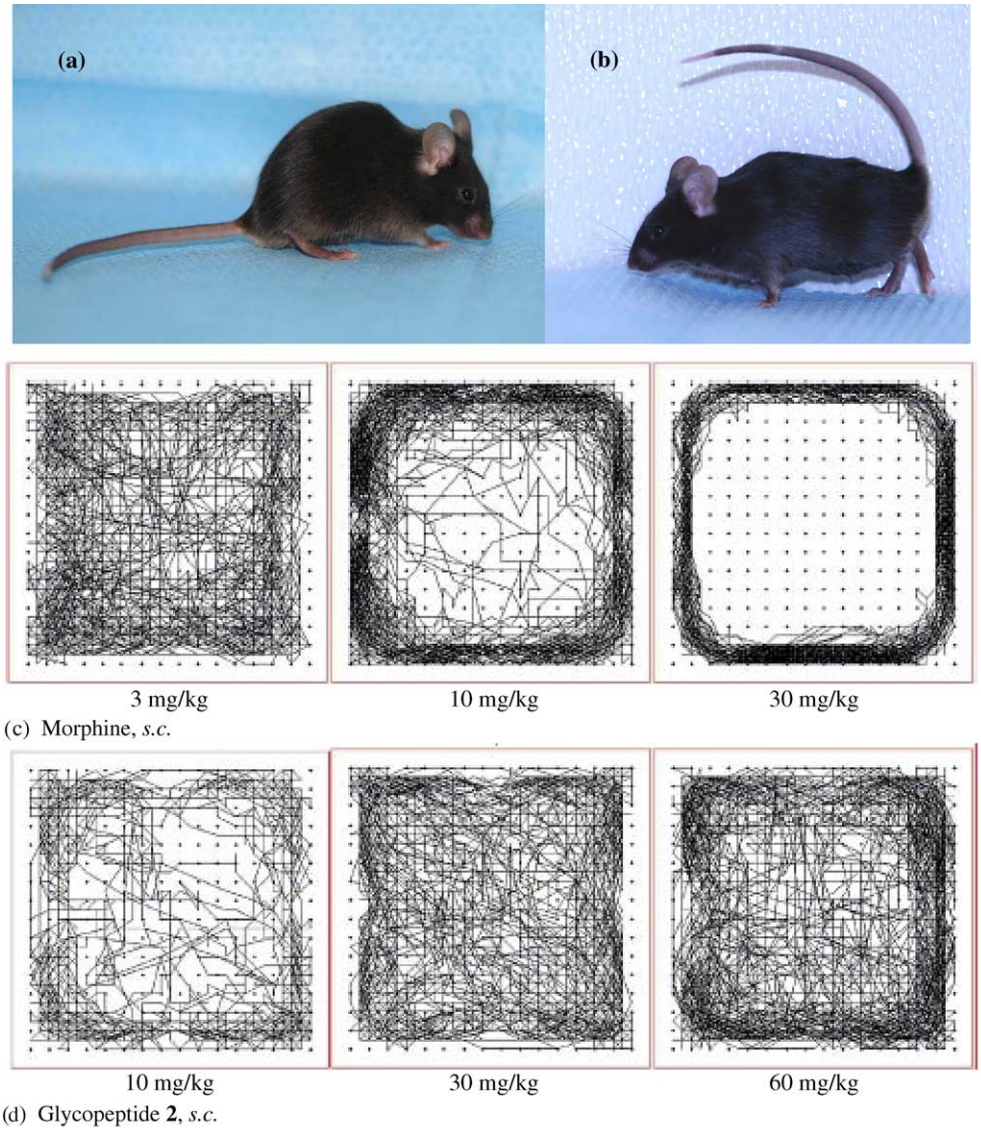

Figure 5. Locomotor effects of opioid drugs on mice. Both mice have received equi-analgesic (A_{90}) doses of drug. (a) Glycopeptide-based analgesia did not induce increased locomotor activity, stereotypical circling, or Straub tail. (b) Morphine-induced analgesia induced large increases in locomotor activity, stereotypical circling and Straub tail. Increased locomotion and stereotypical circling induced by morphine (c), compared to equi-analgesic doses of glycopeptide (d).

pathic.[24] These helical sequences possess hydrophobic and hydrophilic parts, either by primary structure (highly hydrophilic N-terminus and hydrophobic C-terminus), or by secondary structure, with polar residues pointing one to face and the non-polar residues on the opposite face. This allows them to 'float' in a cell membrane, exposing the hydrophilic side to the aqueous exterior of the cell and the hydrophobic side to the lipophilic membrane.[25,26] This peptide–membrane interaction is believed to be important for two reasons.

First, the amphipathic nature of the helix can help guide a drug or hormone to its specific receptor by narrowing the receptor search from a 3-dimensional search to one in 2-dimensions. Surface-assisted 'reduction-of-dimensionality' calculations, performed by Polya in 1921, were examined by Max Delbrck in which he quantitatively demonstrated the viability of this theory.[27] Assuming that no other forces are at work (e.g., convection), and that the membrane is fluid, the probability of a substrate finding its corresponding receptor is much better in 2-dimensions (e.g., a cell surface) than in 3-dimensions (e.g., in solution)—almost 100% effective when the search is reduced to 2-dimensions.

Second, membrane insertion may allow the portion of the peptide or glycopeptide that interacts with the receptor (pharmacophore or 'message') to be fixed in a specific geometry. By restricting mobility in the membrane near the binding site, the amphipathic α-helix can dramatically alter the peptide–receptor interaction.[28] In addition, membrane insertion can also induce a specific conformation in the ligand, different from its solution conformation. It seems clear that the bioactive

conformation of a peptide is the membrane-bound conformation, and that membrane insertion is actually the first step in receptor activation.

Simply producing highly amphipathic sequences is not enough to promote systemic delivery and penetration of the BBB, however. The glycopeptide sequence must also be capable of assuming a water-soluble random coil conformation, and the barrier between the two states must be low enough for rapid interconversion between the two states. In other words, the glycopeptides must show biousian behavior. The first and second generation endorphin analogues **7**–**13** were highly amphipathic, but did not show acceptable transport behavior (Table 2).

The endogenous neuropeptide β-endorphin is a 31-residue naturally occurring opioid peptide. The first five residues of β-endorphin are identical to Met-enkephalin. It has been shown that the α-helical structure of the C-terminal region of β-endorphin plays a role in the receptor binding and opiate activities, and resistance to proteolysis.[29] Kaiser and co-workers[30] proposed that β-endorphin consists of the [Met^5]-enkephalin peptide sequence at the N-terminus, a hydrophilic linker region from residues 6–12, and an amphiphilic helical region between the residues Pro[13] and Gly,[30] which were assumed to be 'helix breakers'. This hypothesis has been supported by the conformational analysis of a number of β-endorphin mimics with artificial C-terminal helical regions with amphipathic character.[31–33] All of the analogues were α-helical by CD measurements, as the monomer or oligomers, and showed strong opioid agonism in vitro when compared to natural β-endorphin. These studies clearly suggest that amphipathicity of the entire peptide is more important than the identity of specific amino acids present in the helical amphipathic C-terminus.[7] This has been further supported by the work by Kyle,[33] who synthesized several potent peptide analogues containing the α-helix-promoting residues α-aminoisobutyric acid (Aib) and N-methyl alanine (MeAla) near the C-terminal region of nociceptin, the natural ligand for the recently identified opioid receptor-like 1 receptor (ORL-1). According to Schwyzer,[7] the N-terminal 'message' is steered toward certain receptors and away from others by the C-terminal 'address' segment, which interacts with the membrane to orient the message with respect to the receptor.

The first- and second-generation endorphins also bore the δ-selective YdGFL∼ opioid message. Formed by simple truncation, the first generation helices, **7**–**10**, were designed to probe the minimum length for helix formation. Essentially, we overshot the target, and all of these compounds were extremely helical, but they were not water soluble, with the exception of helix **8**. This compound possessed appreciable antinociceptive activity, however.[36] All of these compounds were quite soluble in the presence of SDS micelles, however. Since these compounds are so stable in their helical form, that they probably form aggregates, and fall out of solution in the absence of the detergent. The second generation helices, **11**–**13**, were designed to be less lipophilic, and consequently were more water soluble, and showed much less helicity in the presence of micelles[34] (Table 3).

The third-generation helical endorphin-based glycopeptides, **14**–**16**, used the same δ-selective peptide DTLET first studied by Roques, and showed much superior properties, both in the chemistry lab and in the mouse. Using in situ methods in the mouse, not rat studies as before, Egleton was able to measure BBB transport rates independently of analgesia. Initial studies with these glyco-hexadecapeptides indicated that BBB transport rates were determined by the amphipathic nature of the glycopeptides,[35,36] rather than the lipophilicity of the compound, per se,[37] and that they actually show BBB transport rates that are similar to, or even better than the shorter enkephalin analogues.

These endorphin analogues all have the same N-terminal YdGFL∼ opioid message contained in the enkephalin analogues **1**–**6**, and the same C-terminal amide address sequence ∼NLBEKALKS*L-CONH$_2$, where B is the helix-stabilizing α-aminoisobutyric acid (Aib)

Table 2. Helicity of first- and second-generation glycosylated endorphin analogues

Helix glucoside	Glycopeptide sequence	Retention time (RP-HPLC)	% Helicity (CD)	icv Analgesia IC_{50} (pmol)
7	YtGFLGELAS*KWFNALE	8.85 min	69	Insoluble
8	YtGFLGELAS*KWFNALES*	7.95	55	270
9	YtGFLGELAS*KWFNALES*F	9.91	53	Insoluble
10	YtGFLGELAS*KWFNALES*FW	12.48	68	Insoluble
11	YtGFLGLLKS*FAES*WS*NF	6.69	34	∼30
12	YtGFLGKS*FAELWS*NFLS*	5.35	14	∼30
13	YtGFLGLLKS*FWES*WS*NF	8.25	37	∼30

Table 3. Helicity and opioid binding of third-generation glycosylated endorphin analogues

Helix glucoside	Glycopeptide sequence (third generation)	Retention time (RP-HPLC)	% Helicity (CD)	MVD IC_{50} (nM)	GPI IC_{50} (nM)
14	YtGFL(**P**)NLBEKALKS*L-CONH$_2$	31.57	21	34.5	63.1
15	YtGFL(**βA**)NLBEKALKS*L-CONH$_2$	33.50	26	23.0	354
16	YtGFL(**GG**)NLBEKALKS*L-CONH$_2$	30.30	14	18.8	196
—	Morphine	—	—	258	54.7

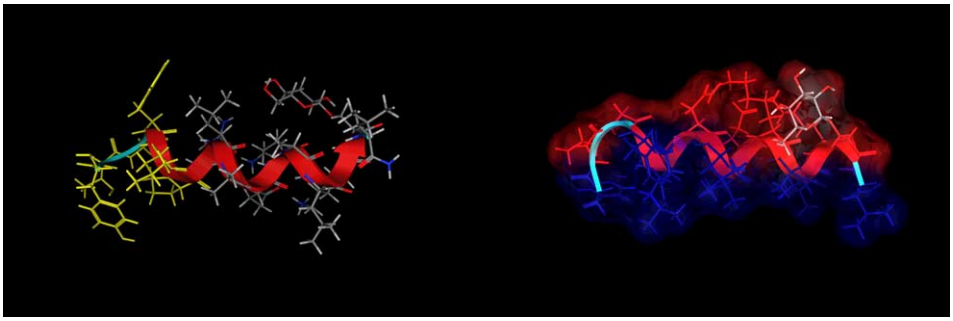

Figure 6. Lipid bound helix. One structure of glycopeptide **14** in the presence of SDS micelles, as determined by NOE-constrained molecular dynamics calculations using MacroModel® v 8.0. The message segment is labeled in yellow, and the helix indicated with the overlaid ribbon. The structure on the right has the hydrophobic (blue) and hydrophilic (red) surfaces labeled. The Connoly surfaces were rendered with the MOE® software package.

residue, and S* is the serine glucoside residue. The 'linker region', which is intended to 'break' the helix, and prevent propagation of the helical address into the opioid message, is different in the three glycopeptides: **14** ⇒ proline, **15** ⇒ β-alanine, and **16** ⇒ glycylglycine.

NMR evidence, in conjunction with NOE-directed Monte Carlo calculations shows that the glycopeptides bind to micelles, and adopt a very restricted set of conformations. For the helices **14**, **15**, **16**, and the disaccharide **17** we see membrane-bound conformational ensembles that are very amphipathic and very helical (Fig. 6), although the degree of helicity varies considerably with the solvent media (Fig. 7). Structural changes in the glycopeptide structure as a function of media are immediately apparent in the NMR spectra. Use of trifluoroethanol (TFE) shows the helical propensity of a peptide sequence, but is not as predictive of the membrane-bound structure. Superior results are achieved with bicelles, which have a true bilayer in the central disk, have much less curvature, and mimic the biological membrane surface more accurately (Fig. 8).

Further information is obtained by the use of plasmon-waveguide resonance (PWR) that provides true dissociation constants (K_D values) for glycopeptide–membrane interactions (Fig. 9). The interaction of the glycopeptide **14** with the lipid bilayer follows a biphasic process, producing an initial shift in the spectra to higher angles (data not shown), followed by a small shift to smaller angles occurring in the order of minutes. This indicates a two-step process, perhaps involving an initial association of the peptide with the bilayer (resulting in a mass increase), followed by a (partial) insertion of the peptide resulting in expulsion of lipid into the Gibbs border that anchors the bilayer to the PWR resonator.[38] The final

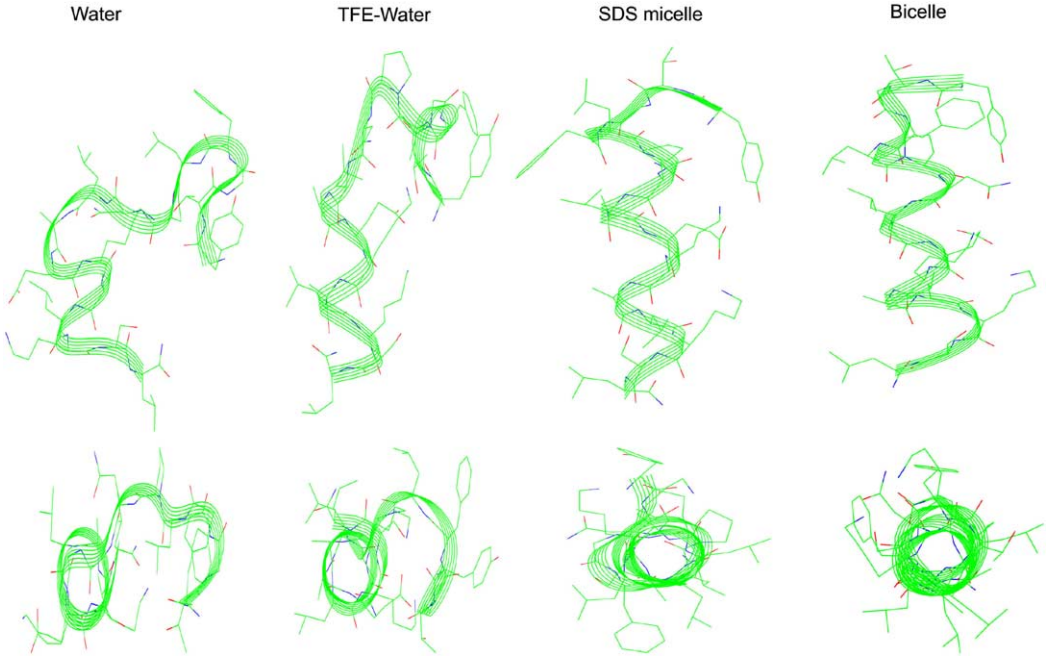

Figure 7. Solvent effects on helicity. Glycopeptide **14** in the presence of H_2O, H_2O–30% TFE, SDS micelles, and zwitterionic bicelles. The carbohydrate residue has been removed from each structure for clarity. A bicelle seems to be a more relevant model for the membrane surface than a micelle that has much more curvature.

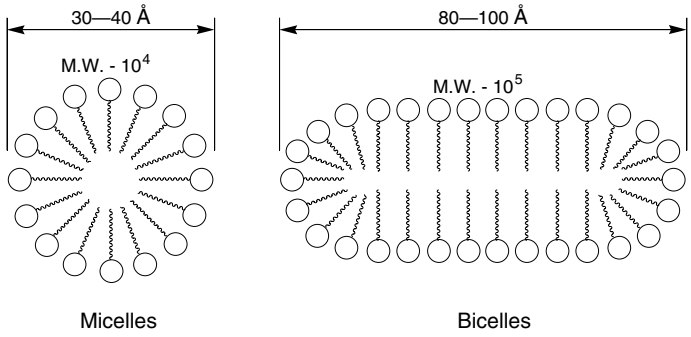

Figure 8. Micelles versus bicelles. Bicelles have much less membrane curvature than micelles, and are more predictive of the membrane-bound glycopeptide structure.

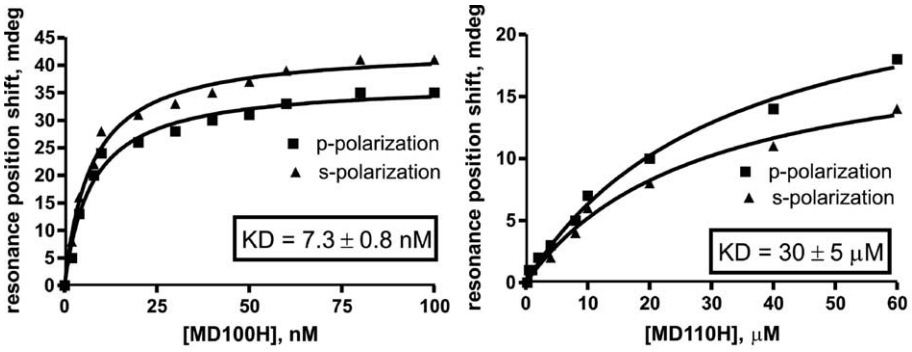

Figure 9. Plasmon-waveguide resonance studies of **14** and **16**. The binding curves are shown for the interaction of peptide MD100H **14** (left) and MD110H **16** (right) with a lipid bilayer formed from hen egg PC. The dissociation constant given was calculated by fitting the data through a single hyperbolic function. The membrane binding correlates strongly with in vivo BBB transport data.

resonance position shifts can be plotted for the incremental additions of glycopeptide to the lipid bilayer and fitted through a hyperbolic function to provide affinity constants (expressed as dissociation constants, K_D). The K_D value for glycopeptide **14** was determined to be in the nanomolar range, whereas glycopeptide **16** bound in the micromolar range (Fig. 9) with much smaller spectral shifts observed even at μM concentrations of the glycopeptide in the cell sample. The spectral changes observed with **16**, contrary to what was observed with **14**, follow a monophasic transition in a somewhat slower process (data not shown).

Both glycopeptides possess the same glycoside moiety near the C-terminus of the identical C-terminal address sequence, and possess the same N-terminal message sequence. Despite this, the two glycopeptides show very different affinities for the bilayer, suggesting that glycosylation is not responsible for this difference in the degree of membrane interaction, and rather that the

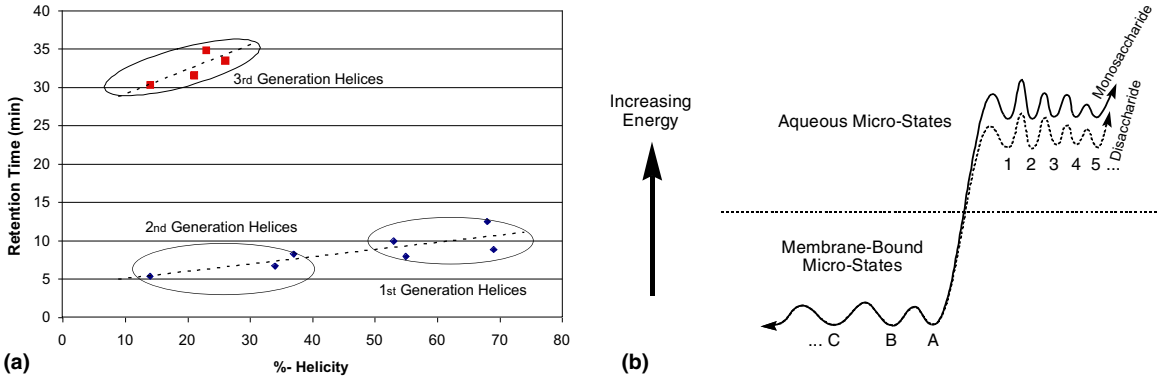

Figure 10. Helicity is responsible for amphipathicity and biousian behavior. (a) The reversed-phase HPLC retention time in minutes plotted versus the ellipticity per residue for a series of amphipathic glycopeptides. (b) A hypothetical energy level diagram illustrating a hypothetical monosaccharide and disaccharide. The glycopeptide can exist as a series of (water soluble) random coil conformations, or as a more restricted series of (membrane bound) amphipathic helix conformations. Increasing the degree of glycosylation will stabilize the water-soluble conformations, and alter the biousian state.

linkage region (**14** ⇒ Pro vs **16** ⇒ Gly-Gly) changes the peptide backbone structure, thus altering the amphipathicity of the two molecules. Further studies with the unglycosylated peptides will shed more light on this issue.

Glycopeptide **14** has been found cross the blood–brain barrier (BBB) very efficiently in mice, whereas **16** did not (Fig. 11). The capability of glycopeptide **14** to cross the BBB may be correlated with its high affinity for the lipid bilayer. The PWR data suggest that the glycopeptides do not insert into the lipid bilayer in a perpendicular fashion. Furthermore, neither peptide causes significant lysis of the bilayer, because no significant changes in the resonance shape are caused by the peptide binding. We propose that the glycopeptide **14** promotes negative membrane curvature at the endothelial cell wall, and that the negative curvature leads to increased rates of endocytosis, which in turn results in enhanced BBB transport via transcytosis (Fig. 10).

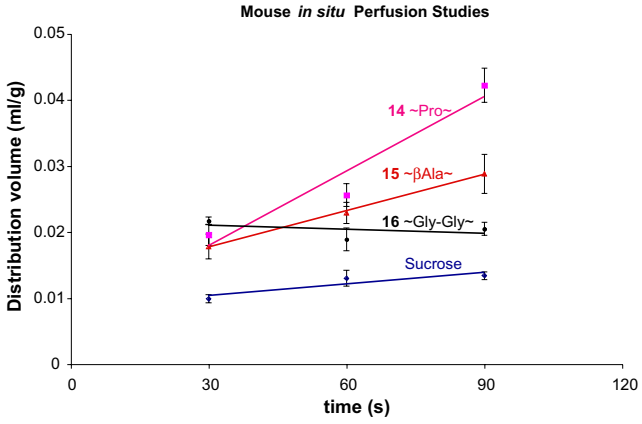

Figure 11. Mouse BBB transport data for glycopeptides **14**, **15**, and **16**. Sucrose is a marker that does not penetrate the BBB, used to insure that the BBB remains intact during the measurements. BBB penetration is given by the slope of the curve, and the initial volume of distribution given by the *Y* intercept. BBB transport correlates strongly with the strength of the glycopeptide–membrane interaction (Fig. 9).

4. Conclusion

Amphipathicity is controlled by the amount of time the molecules spend in a helical conformation, which can be measured by interaction the amount of time spent in association with a lipid surface (HPLC retention time), or by the degree of association with micelles (ellipticity measured by circular dichroism). The fact that these two apparently diverse measurements show a linear correlation suggests that they are both dependent on the same underlying property (e.g., biousian character). Two conformational ensembles exist for the biousian glycopeptides. A relatively small number of low energy membrane-bound microstates compose the membrane-bound ensemble, and a much larger number of aqueous microstates compose the water-soluble ensemble, which is of higher energy (i.e., the glycopeptides prefer to be associated with the membrane). Thus, for any given amphipathic helix with a given affinity for the membrane, there should be a corresponding carbohydrate moiety of sufficient affinity for the aqueous compartment that will balance the affinity for the membrane, leading to the ability to leave the membrane surface and 'membrane hopping'. If this hypothesis is true, then glycopeptide **16** does not have sufficient affinity for the membrane to undergo endocytosis.

While there is still much to be learnt about both the details of the transport process, and the binding of the amphipathic glycopeptides, an important principle has emerged concerning transport. It seems clear that for effective drug delivery and BBB transport one must have a biousian glycopeptide that essentially has two states: (1) a state defined by one or more membrane-bound conformations that permit or promote endocytosis; (2) a state defined by a water-soluble, or random coil state that permits 'membrane hopping'. The key to efficient transport is to balance these two states so that the compound is neither retained in the membrane, nor held in aqueous solution so that it cannot undergo adsorptive endocytosis. It is possible that aggregation of glycopeptides on a membrane surface actually initiates and promotes endocytosis.

The reduced intensity of non-analgesic side effects produced by the glycosyl enkephalin analogues suggest that several of these compounds (e.g., **2**, **5**, and **6**) have the potential to replace morphine in many clinical applications where morphine and classical narcotic analgesics are contraindicated.[34] Taken as a whole, these results demonstrate that judicious choice and placement of glycosides on neuroactive peptides can result in penetration of the BBB and potent CNS activity (analgesia in this case) after peripheral administration. Glycopeptides related to endorphin have also been rendered permeable to the BBB using this same glycosylation strategy,[39] thus opening a new mode of drug design, based not on plant-derived alkaloids, or on chemical serendipity, but on endogenous neuropeptides from the human brain. Complete absorption, metabolism, and excretion studies (ADME) need to be completed, and oral bioavailability needs to be explored. The fact that the glycosylation strategy seems to be effective with the much larger endorphin analogues (e.g., compounds **14** and **15**) suggest that this approach may have general applicability to BBB transport of non-analgesic (or even non-opioid) neuropeptides, which could lead to novel treatments for a wide range of neurological disorders.

5. Experimental

5.1. Glycopeptide synthesis and purification

Amino acids, coupling reagents and Rink-amide resin were purchased from Advanced ChemTech (Louisville, USA). The per-acylated Fmoc amino acid glycosides were synthesized using previously published methods.[2] The glycopeptides were synthesized manually by standard solid-phase Fmoc (fluorenylmethoxycarbonyl) methods employing on Rink-amide resin.[40] The side

chain protected amino acids used in the synthesis were Fmoc-Lys(Boc)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-DThr(But)-OH, and Fmoc-Tyr-(But)-OH. The amide couplings were with HBTU/HOBt/DIPEA. Each coupling was performed in a manual peptide synthesis vessel using DMF as solvent with N_2 agitation for 90 min. The coupling was monitored by the Kaiser ninhydrin test. Fmoc groups were removed with a solution of 20% piperidine in DMF. Once the glycopeptide was assembled and the final Fmoc group was removed, the –OAc protecting groups were cleaved from the carbohydrate with 80% $H_2NNH_2{\cdot}H_2O$ in CH_3OH. The glycopeptide was then cleaved from the resin with a cocktail $F_3CCOOH/Et_3SiH/H_2O/PhOMe/CH_2Cl_2$ (9:0.5:0.5:0.05:1), which also removed the side chain protecting groups. The crude glycopeptides were precipitated with ice-cold ether, filtered, redissolved in H_2O, and lyophilized. The glycopeptides were purified by RP-HPLC with a preparative RP(C-18) column using CH_3CN/H_2O gradient containing 0.1% TFA. Homogeneity of the final glycopeptides was assured by analytical RP-HPLC and mass spectrometry.

5.2. Plasmon-waveguide resonance (PWR) spectroscopy

The PWR instrument used for these experiments was Aviv Beta prototype version device obtained from Proterion Corp. (Piscataway, NJ) having a spectral resolution of 1 millidegree. Self-assembled solid-supported lipid membranes were prepared according to the method used for the formation of freely suspended lipid bilayers.[41] This involves spreading a small amount of lipid solution across an orifice in a Teflon sheet that separates the thin dielectric film (SiO_2) from the aqueous phase. The hydrophilic surface of hydrated SiO_2 attracts the polar groups of the lipid molecules, thus inducing an initial orientation of the lipid molecules, with the hydrocarbon chains pointing toward the droplet of excess lipid solution. The next steps of bilayer formation, induced by adding aqueous buffer to the sample compartment of the PWR cell, involve a thinning process and the formation of a plateau-Gibbs border of lipid solution that anchors the membrane to the Teflon spacer. In the present experiments, the lipid films were formed from a solution containing 5 mg/mL egg phosphatidylcholine (PC) in squalene/butanol/methanol (0.05:9.5:0.5, v/v). The lipid was purchased from Avanti Polar Lipids (Birmingham, AL). All experiments were carried out at constant temperature of 25 °C, using 10 mM Tris buffer containing 0.5 mM EDTA and 10 mM KCl (pH = 7.3), in the 1-mL sample cell. Aliquots of the glycosylated peptides, dissolved in deionized water, were injected stepwise in the PWR cell sample and the signal monitored until equilibrium was reached (PWR signal steady). Finally, dissociation constants (K_D values) were obtained by plotting the resonance minimum position for the PWR spectra as a function of peptide concentration in the cell sample, and fitting the data to a simple hyperbolic function to describe the binding of a ligand to a lipid bilayer. Data analysis was performed with GraphPad Prism (GraphPad Software Inc., CA, USA).

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