

Glycosylation Improves the Central Effects of DAMGO

John J. Lowery¹, Larisa Yeomans², Charles M. Keyari², Peg Davis³, Frank Porreca³, Brian I. Knapp⁴, Jean M. Bidlack⁴, Edward J. Bilsky¹ and Robin Polt^{2,*}

¹Department of Pharmacology, University of New England, 11 Hill Beach Rd., Biddeford, Maine 04005, USA

²Department of Chemistry, The University of Arizona, 1306 E. University Blvd, Tucson, AZ 85721, USA

³Department of Pharmacology, Arizona Health Sciences Center, Tucson, AZ 85726, USA

⁴Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, NY 14642, USA

*Corresponding author: Robin Polt, polt@u.arizona.edu

A series of μ -agonist DAMGO analogs were synthesized and pharmacologically characterized to test the 'biousian' hypothesis of membrane hopping. DAMGO was altered by incorporating moieties of increasing water solubility into the C-terminus via carboxamide and simple glycoside additions. The hydrophilic C-terminal moieties were varied from glycinol in DAMGO (1) to L-serine amide (2), L-serine amide β -D-xyloside (3), L-serine amide β -D-glucoside (4), and finally to L-serine amide β -lactoside (5). Opioid binding and mouse tail-flick studies were performed. Antinociceptive potency (intravenous) increased, passing through a maximum ($A_{50} \approx 0.2 \mu\text{mol/kg}$) for 2 and 3 as membrane affinity versus water solubility became optimal, and dropped off ($A_{50} \approx 1.0 \mu\text{mol/kg}$) for 4 and 5 as water solubility dominated molecular behavior. Intravenous A_{50} values were plotted versus hydrodynamic values (glucose units, g.u.) for the glycoside moieties, or the hydrophilic/hydrophobic Connolly surface areas (A_{50} versus $e^{-A_{\text{water}}/A_{\text{lipid}}}$), and provided either a V-shaped or a U-shaped curve, as predicted by the 'biousian' hypothesis. The μ -selective receptor profile was maintained (K_i 's = 0.66–1.3 nM) upon modifications at the C-terminus. The optimal 'degree of glycosylation' for the DAMGO peptide message appears to be between 1.25 and 1.75 g.u. (hydrodynamic g.u.), or 0.75 and 0.90 in terms of the surface-derived amphipathicity values.

Key words: amphipathicity, antinociception, blood–brain barrier, biousian, DAMGO, drug delivery, enkephalin, glycopeptide

Received 24 October 2006, revised 15 December 2006 and accepted for publication 16 December 2006

The discovery of endogenous opioid ligands (1) and their receptors (2) gave an early impetus to the concept of drugs for the central nervous system (CNS) based on peptide neurotransmitters. In principle, peptides could replace naturally occurring alkaloids such as morphine or codeine, or petroleum-derived drugs such as fentanyl. Unfortunately, this impetus was quickly dampened as pharmacologists encountered the many problems associated with the synthesis (3), binding (4), stability (5), and biodistribution (6) of peptides. Over the intervening decades, most of these problems have been addressed, and the prospects for peptide-based drugs once again seem bright (7). One of the last problems to be addressed is the penetration of the blood–brain barrier (BBB; 8). Typical peptide neurotransmitters have MWs > 500, log P_s < –2.0, and many more H-bonds than Lipinski's rules of transport would allow (9). In fact, the very features that make peptides useful as neurotransmitters make them violate nearly all of the transport rules for typical pharmaceutical compounds. Our studies with glycosylated enkephalins have given rise to the concepts of *membrane hopping* (10) and the *biousian hypothesis* (11).

Essentially, neurotransmitters possess highly amphipathic conformations that promote strong interactions with membranes (12–14). Thus, in a typical CNS situation a neurotransmitter (e.g. Met-Enkephalin or Leu-Enkephalin) is released from a presynaptic vesicle, requiring it to travel only several hundred angstroms at most (the distance between the presynaptic and postsynaptic membrane), where it can adsorb strongly to the postsynaptic neuronal membrane that in turn facilitates rapid binding of the neurotransmitter to a membrane-bound receptor (e.g. MOP; Figure 1).

The attachment of a glycoside or another water-soluble moiety (e.g. a cationic amino acid residue, such as the arginine in TAPA, Tyr-D-Arg-Phe- β -Ala-OH; 14) in the appropriate position can, in principle, lead to increased stability of the aqueous state *without perturbing the membrane-bound conformation of the peptide message*. Thus, instead of simply binding to a biologic membrane, the glycopeptide can 'hop off' the membrane, free to travel some distance before it encounters another membrane that will permit the glycopeptide to 'hop on' again. By carefully balancing the free energy of the two states (e.g. membrane-bound state versus aqueous state, Figure 2), the optimal amount of time will be spent on the

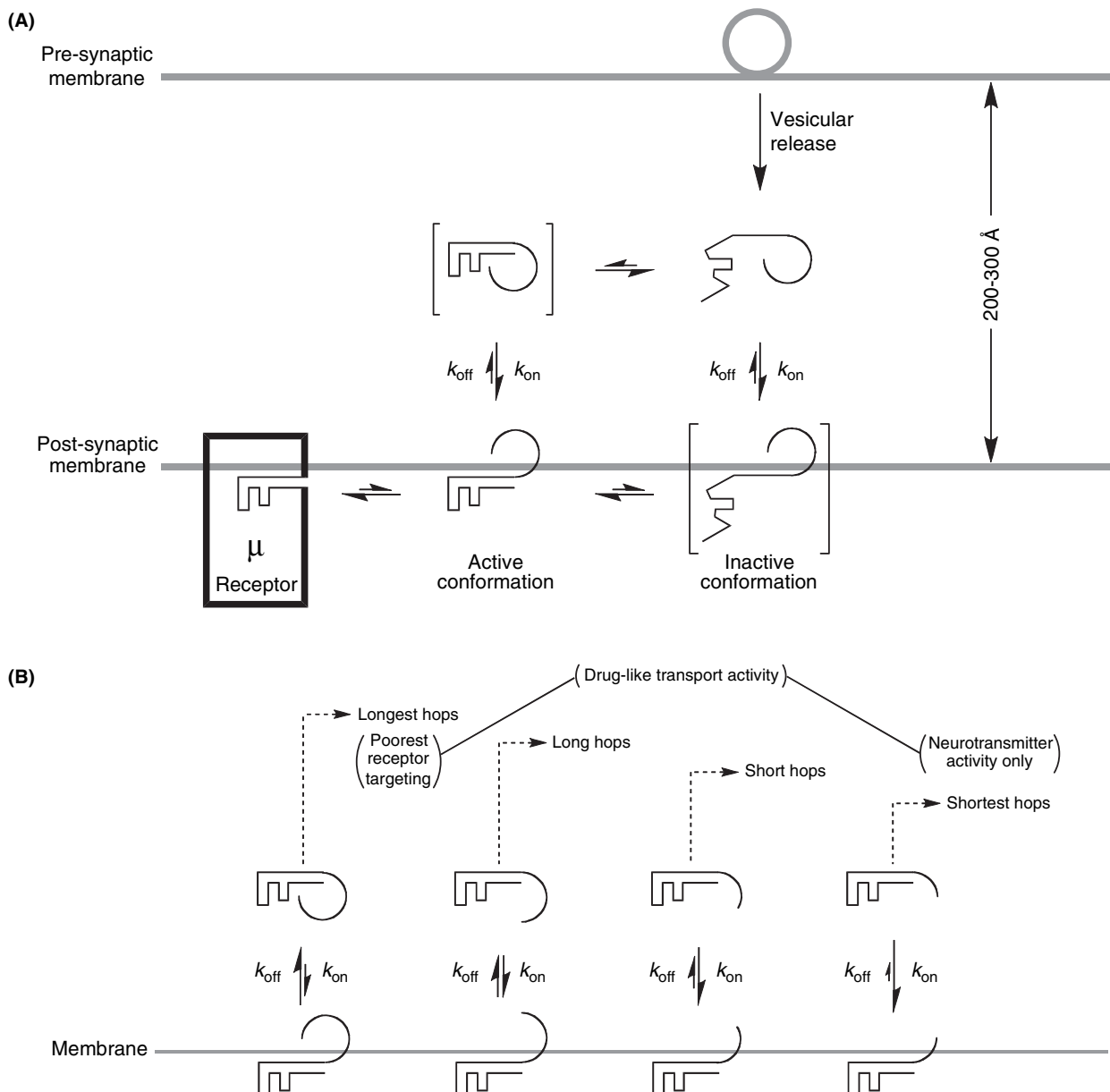


Figure 1: Peptide neurotransmitters strongly associated with membranes. (A, above) After release from the presynaptic neuron, peptide neurotransmitters (e.g. enkephalins) strongly associate with the postsynaptic membrane ($k_{on} \gg k_{off}$) and bind to a G-protein coupled receptor (μ -opioid receptor or MOR) via a membrane-bound conformation (Fisher's lock & key). Previous studies show that active conformations are favored in the membrane and inactive conformations are favored in the absence of a membrane. Incorporation of glycosides to optimize membrane hopping. (B, below) Incorporation of a glycoside moiety, represented by the 270°, 180°, 120° and 90° arcs, shifts the k_{on}/k_{off} equilibrium to facilitate 'membrane hopping'. Drug-like activity results when the k_{on}/k_{off} ratio is near ideal. Previous studies show that membrane-bound (active) conformations of glycosylated enkephalins differ from their aqueous (inactive) conformations (1,2).

membrane for binding and endocytosis versus the amount of time spent moving through the aqueous compartments *in vivo*. This is the heart of the bioussian hypothesis: two 'ousia' (essences or substances) potentially exist within the same molecule. In this way, peptide neurotransmitters can be converted from compounds that spend most of their time associated with membranes into drug-like molecules that more freely diffuse throughout the aqueous compartments of the biologic organism.

Methods and Materials

To further explore and exploit the bioussian hypothesis, the classical μ -selective agonist DAMGO (**1**; 15) was used as a lipophilic peptide 'message' (16). It is generally agreed that the μ -receptor is responsible for the bulk of the antinociceptive effects of opioid agonists and as the majority of opioid analgesics that have been studied or used clinically are μ -agonists, it was thought that the exploration

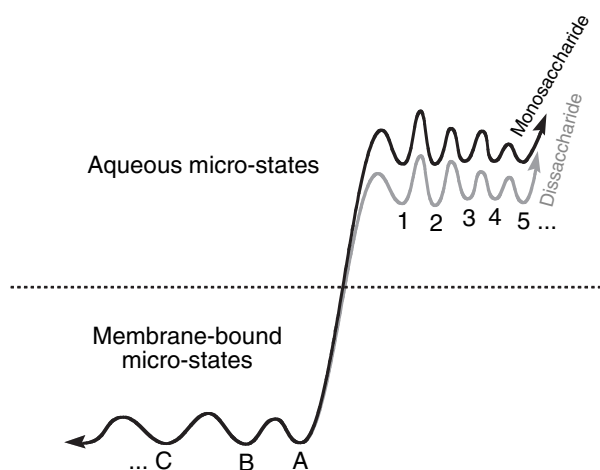


Figure 2: Energetics of aqueous versus membrane-bound states. Additional carbohydrate residues are postulated to decrease the energy of the aqueous conformational ensemble (1, 2, 3, 4, 5...) without substantially affecting the membrane-bound ensemble (A, B, C...).

of the biosian hypothesis within the context of a pure μ -agonist would simplify interpretation of the results. Modification of the parent peptide **1** included the addition of moieties that increased the water-soluble 'address' segment of the molecule (Figure 3). The peptide **2** and glycopeptides **3–5** were synthesized using published Fmoc methods with O-(1H-benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate/diisopropylethylamine (HBTU/DIEA) and Rink amide resin on a Protein Technologies, Inc. (Tucson, AZ, USA) PS3 synthesizer (3).

Radioligand-binding studies

Binding was determined in Chinese hamster ovary (CHO) cell membranes expressing either the human μ -, δ -, or κ -opioid receptors (MOR, DOR, and KOR). Cells were incubated with 12 concentrations of glycopeptide and the indicated radiolabeled ligand (Table 1). Non-specific binding was measured by inclusion of 10 μ M naloxone.

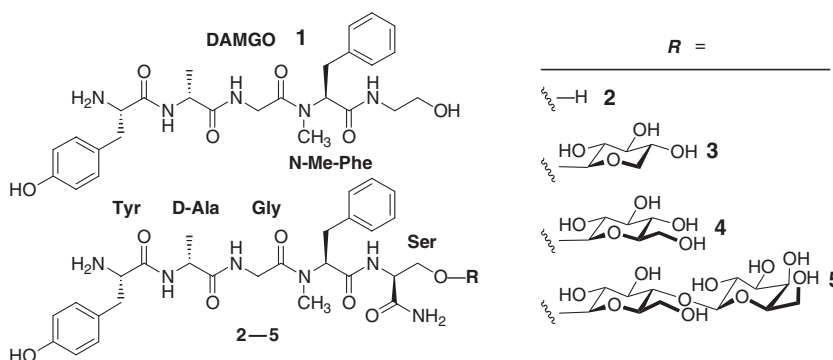


Figure 3: DAMGO analogs. Increasing water solubility is introduced via the carboxamide group (**2**), carboxamide + β -xyloside (**3**), carboxamide + β -glucoside (**4**), and carboxamide + β -lactoside (**5**) added to DAMGO (**1**). The peptide 'message' consists of Tyr-D-Ala-Gly-(N-Me)Phe. The ethanolamine (glycinol) is assumed to have a hydrodynamic value = 0.25 glucose units (g.u.), carboxamide group = 2 g.u., and the saccharides the accepted values, β -D-xylose = 0.5, β -D-glucose = 1, and β -lactose = 2 g.u., respectively.

Data are the mean K_i values \pm SEM from three experiments performed in triplicate.

Animal and injections

All *in vivo* studies used adult male ICR mice (25–30 g; Harlan Industries, Cleveland, OH, USA) that were maintained on a 12 h light/dark cycle (lights on at 07:00 hours) in a temperature and humidity-controlled animal colony. All testing was carried out between 10:00 and 15:00 hours. Studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

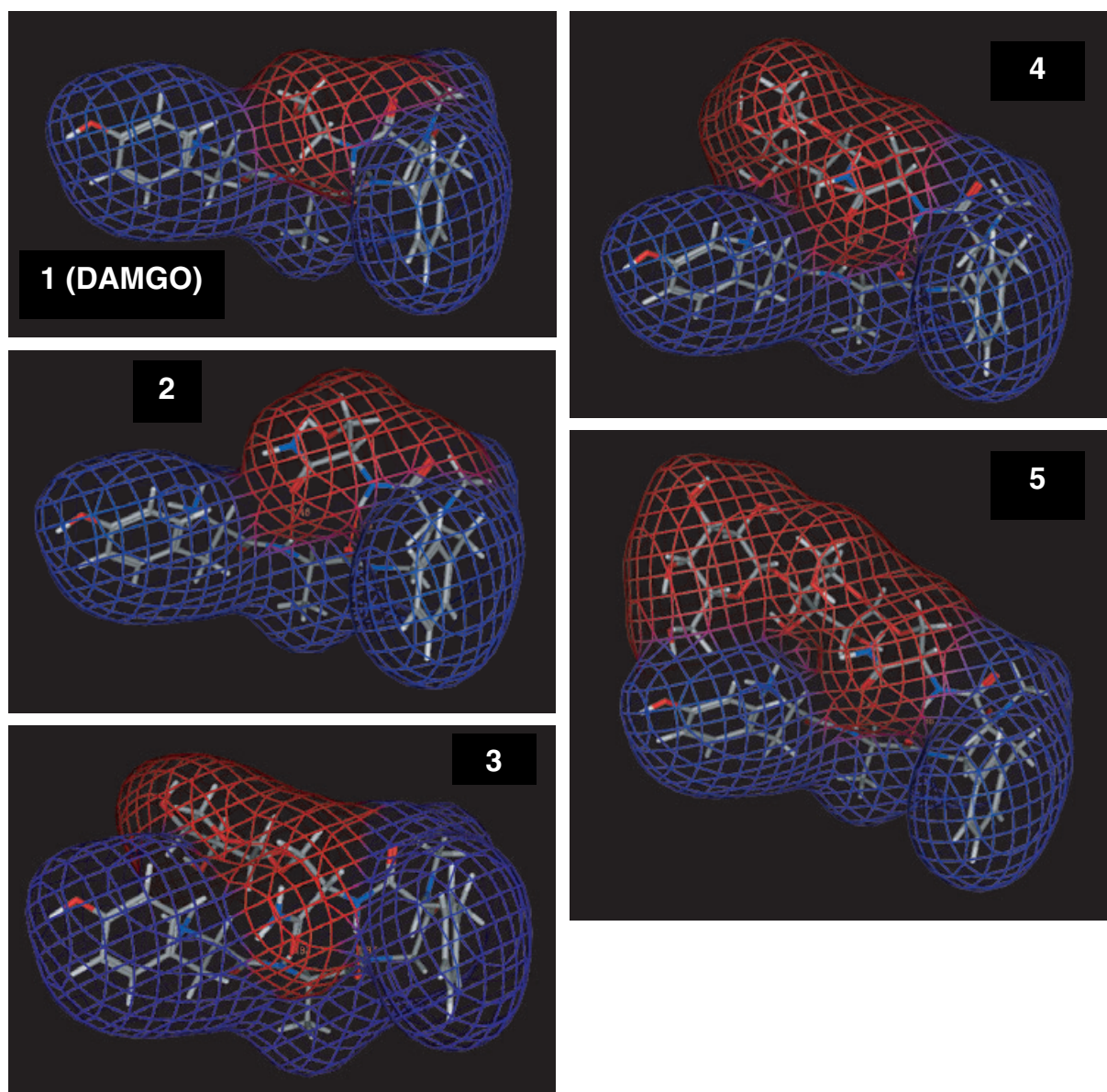
For intracerebroventricular (i.c.v.) injections, mice were lightly anesthetized with ether and an incision was made in the scalp. Injections were performed using a 10 μ L Hamilton microsyringe (Hamilton Company, Reno, NV, USA) at a point 2 mm caudal and 2 mm lateral from bregma. Compounds were injected at a depth of 3 mm in a volume of 5 μ L. Intravenous (i.v.) injections were performed by restraining the mouse in a Plexiglas holder, dipping the tail for 10 seconds in 40 $^{\circ}$ C warm water to dilate the tail vein, and subsequent injection into the vein with a 30-gauge needle. All compounds were dissolved in distilled water (i.c.v. injections) or physiologic saline (i.v. injections).

Antinociceptive testing and statistics

Antinociception was assessed using the 55 $^{\circ}$ C warm water tail-flick test. The latency to the first sign of a rapid tail-flick was taken as the behavioral end-point. Each mouse was first tested for baseline latency by immersing its tail in the water and recording the time to response. Mice not responding within 5 seconds were excluded from further testing (average latency = 2.1 seconds). Mice were then administered the test compound and tested for antinociception at 10, 20, 30, 45, 60, 90, 120 and 180 min postinjection. A maximum score was assigned (100%) to animals not responding within 10 seconds. Antinociception was calculated by the following formula: % antinociception =

Table 1: Antinociceptive potencies (*i.c.v.* and *i.v.* in mouse 55°C tail-flick assay) and binding affinities for DAMGO and related analogs at MOR, DOR and KOR.

Opioid	A_{50} (95% CI)		K_i (nM) \pm S.E.M.			Ratio μ : δ : κ
	<i>i.c.v.</i> (pmol/ mouse)	<i>i.v.</i> (μ mol/kg)	[3 H]DAMGO MOR	[3 H]Naltrindole DOR	[3 H]U69,593 KOR	
Morphine	2,384 (1,770–3,210)	7.84 (6.07–10.11)	0.79 \pm 0.12	290 \pm 38	12 \pm 1.3	1:370:15
DAMGO 1	30 (20.0–40.0)	1.88 (1.48–2.38)	0.56 \pm 0.006	990 \pm 35	270 \pm 9.3	1:1900:510
2	2.0 (2.0–3.0)	0.20 (0.16–0.26)	0.68 \pm 0.02	600 \pm 44	190 \pm 9.3	1:880:280
3	2.0 (2.0–3.0)	0.27 (0.21–0.32)	1.30 \pm 0.16	730 \pm 66	160 \pm 10	1:560:120
4	19 (12.0–29.0)	0.72 (0.56–0.93)	1.30 \pm 0.14	54% at 10 μ M	270 \pm 2.5	1:>5000:210
5	2.0 (2.0–3.0)	1.15 (0.82–1.64)	0.66 \pm 0.05	1600 \pm 129	350 \pm 51	1:2400:530

**Figure 4:** Calculated Connolly surfaces for the μ -selective DAMGO series. The Connolly surface, calculated in \AA^2 , was divided into two sections, a lipophilic (blue) surface, A_{lipid} , associated with the peptide message segment YaG(N-MeF), and a hydrophilic (red) surface, A_{water} , defined by the address segment.

$100 \times (\text{test latency} - \text{control latency}) / (10 - \text{control latency})$. Dose-response lines were constructed at times of peak agonist effect, and analyzed by linear regression using FLASHCALC software (17). All A_{50} values (95% confidence limits) shown are calculated from the linear portion of the dose-response curve. A minimum of three doses/curve and 8–10 mice were used at each dose level.

Results and Discussion

The binding affinities and antinociceptive potency of **1–5** are summarized in Table 1 (values for morphine sulfate are included for comparison purposes). The binding affinities and receptor preferences of the μ -selective DAMGO derivatives (**2–5**) are similar to the parent compound (0.56–1.3 nM K_i values for MOR, selectivity ratios for MOR over DOR and KOR of >500 and 100, respectively).

In general, the addition of more water-soluble groups in the address segment of DAMGO (**1**) increased the i.v. potency of the compounds (**2**, **3**, and **5**). Surprisingly, compound **4** was significantly less potent than the other three, with a calculated A_{50} value similar to **1**. The potency difference may be due to much lower affinity/efficacy at the DOR or some unique physiochemical property that affects receptor interactions (e.g. ability to interact with opioid heterodimers and homodimers). It should be noted that after i.v. administration this compound had the longest duration of action of any of the compounds tested (>180 min versus 90–120 min). In contrast, the duration of action of glycopeptide **4** after i.v. administration was similar to the other glycopeptides (*AUC* calculations and visual inspection of time-course plots, data not shown).

Based on previously published studies (10,11,15) the i.v. antinociceptive potencies of enkephalin-based glycopeptides is largely determined by their ability to penetrate the BBB by transcytosis (18) which in turn depends on the biosian character of the drugs. One may consider two extremes that result in differential delivery of a peptide drug into the CNS. First, the peptide binds tightly to

biologic membranes and is effectively removed from the solution. Secondly, the peptide remains in aqueous solution, effectively preventing it from interacting with biologic membranes. Thus, the goal in producing glycopeptides that are capable of effective BBB penetration and receptor binding/activation, is to balance the degree of glycosylation, which effectively determines the amount of time the glycopeptide spends in contact with the endothelial membrane of the BBB, as well as other membranes that the glycopeptide is likely to encounter [e.g. the cell membrane in which the G-protein coupled receptor (GPCR) is embedded]. Affinity for the membrane is still required for effective binding to the GPCR (19), but a certain amount of 'membrane hopping' is required for effective drug transport. Thus, if one were to plot the BBB transport or antinociceptive A_{50} values versus the membrane affinity, one would predict a U-shaped or V-shaped curve. Note that $\log P_{o/w}$, or 'size-based' analyses (20) can be useful for prediction of *passive diffusion*, but not for the prediction of endocytotic events.

The amphipathic nature of peptides **1–5** can be visualized by calculation of a Connolly surface (solvent accessible surface) for each amphipathic species using the molecular mechanics package of MOE[®] (Chemical Computing Group, Montreal, QC, Canada) and by labeling the surface blue to indicate the lipophilic portion of the surface and red to indicate the hydrophilic portion of the surface (21; Figure 4). The ratio of the two types of surface areas was used to create an expression of the amphipathicity using the formula $\mathbf{A} = e^{-A_{\text{water}}/A_{\text{lipid}}}$. While the actual amphipathicity of each molecule will vary somewhat as each molecule is flexible and actually exists as an ensemble of conformations, it is not likely that the variation in \mathbf{A} will be large. In any case, it is not likely that the order of amphipathicity will be different than what is predicted by this analysis.

An alternative method of amphipathic analysis was also used. In this approach, hydrodynamic values of the hydrophilic portion of **1–5** were assigned, using accepted glucose unit values (g.u.; 22,23). For compounds **1** and **2** the single primary HO-group was assigned a value of 0.25 g.u. For compounds **2–5** the carboxamide

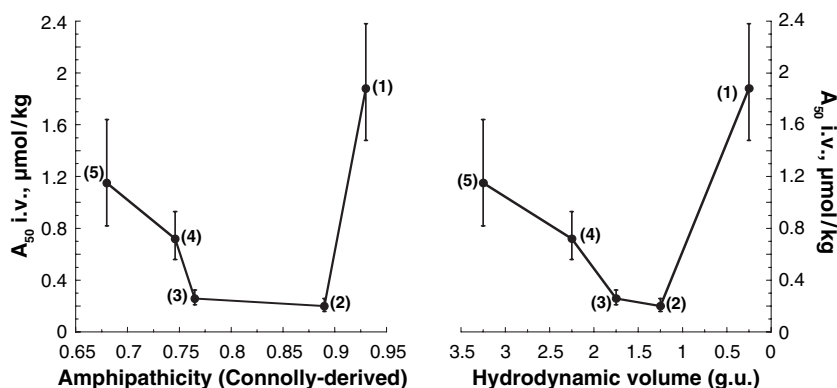


Figure 5: Centrally mediated antinociception shows a U-shaped or V-shaped curve when correlated with two different measures of amphipathicity. The hydrodynamic values (glucose units) or Connolly-derived amphipathicity values are plotted along the X-axes, and A_{50} values (and 95% confidence intervals) derived from mouse intravenous tail-flick data are plotted on the Y-axis. Both analyses produce a U-shape or V-shape, as predicted by the 'biosian hypothesis' (10). The amphipathicity values, \mathbf{A} , were calculated using the formula $\mathbf{A} = e^{-A_{\text{water}}/A_{\text{lipid}}}$, where A_{water} = the Connolly surface area of the hydrophilic moiety (\AA^2) and A_{lipid} = the Connolly surface area of the rest of the lipophilic peptide message segment YaG(N-MeF).

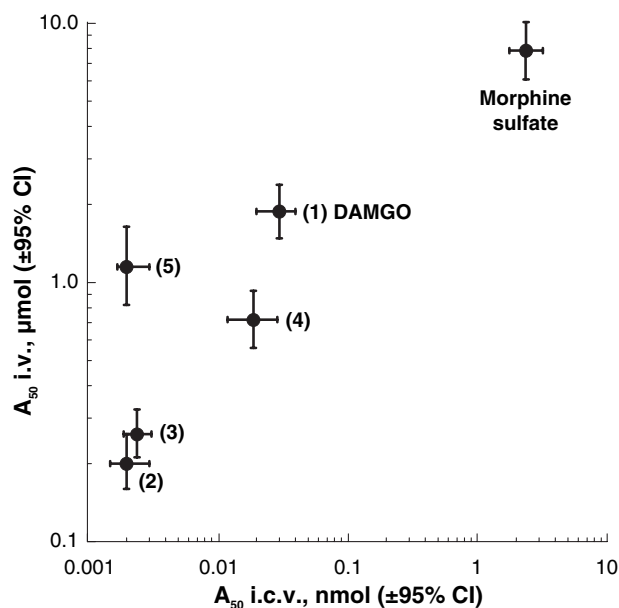


Figure 6: Intravenous (i.v.) versus intracerebroventricular potency. The analgesic potencies (A_{50} values and 95% confidence intervals) are measured in the mouse 55 °C tail-flick assay after i.c.v. administration (horizontal axis, nmol per mouse), and i.v. administration (vertical axis, $\mu\text{mol}/\text{kg}$). Morphine sulfate (μ -agonist) is included as a reference point, but has been excluded from the correlation values.

group (C-terminal amide) was assigned a value of 1.00 g.u. This seems appropriate as the hydrodynamic change from Glc to GlcNAc or Gal to GalNAc is 1.00–2.00 g.u. Both methods of analysis are compared side-by-side (Figure 5), and are plotted versus the i.v. A_{50} values (with 95% confidence intervals). A plot of the i.c.v. antinociception vs the i.v. antinociception leads to a non-linear plot (Figure 6).

In this study, μ -agonists based on the modified enkephalin analog DAMGO (**1**) have been used to help support the 'biosian hypothesis' (10). Opioid neurotransmitters and most peptide-based opioid agonists that have been studied to date are amphipathic and bind tightly to model membranes and (presumably) to biologic membranes *in vivo* (Figure 1). By incorporation of water-soluble 'address segments' into the C-terminus of neurotransmitter-like opioid agonist DAMGO (**1**) to produce peptide **2** and glycopeptide **3**, corresponding increases in the bioavailability of the agonist 'message segment' is observed *in vivo* as indicated by increases in centrally mediated antinociception. We hypothesize that this is due in part to the increased water solubility of the neurotransmitter, which effectively increases the range of action of **1** from a few hundred Å, i.e. the distance across the synaptic cleft, to much larger distances, effectively allowing the peptide message in **2** and **3** to 'hop' from membrane surface to membrane surface, and thereby acquire drug-like properties. As the water solubility is further increased (glycopeptides **4** and **5**), it is suggested that the affinity for the membrane is reduced to the point that interaction with the BBB is reduced (24), effectively reducing the CNS penetration of **4** and **5** and reducing the i.v. antinociceptive potency.

A number of factors determine the activity of a peptide-based drug candidate in the CNS. A primary factor is the bioavailability of the drug to the receptor populations of interest (25). We hypothesize that the biosian behavior of the glycopeptides contributes to the observed potency differences of **1–5** via the ability of the molecules to associate with membranes both within the CNS and the periphery. Based on our previous research with enkephalin-based glycopeptides with mixed μ/δ activity, we also believe that this same biosian behavior may be responsible for the increased transport of the glycopeptides across the BBB (10). Direct transport measurements of **1–5** are in progress, and will help better define the role of membrane hopping within the synaptic cleft and elsewhere. We are also in the process of determining the stability and distribution of molecules **1–5** as these factors can influence antinociceptive potency determinations *in vivo*.

Future Directions

In conclusion, we have synthesized a series of DAMGO analogs that differ in terms of their amphipathic character. The calculations of hydrodynamic values (g.u.) or Connolly-derived amphipathicity values suggest that glycopeptide drugs based on peptide neurotransmitters can be designed that optimize potency (and presumably CNS bioavailability). If these strategies can be extended to other non-opioid peptide transmitters, then this approach will prove useful in advancing peptide-based therapies to diseases where small molecule drugs are not presently available.

Acknowledgments

We thank the Office of Naval Research (Grants 14-02-01-0471 and 14-05-1-0807) and the National Science Foundation (CHE-607917) for support. None of this work would have been possible without the enormous contributions of the late Professor R. Bruce Merrifield (1922–2006).

References

1. Lord J.A.H., Waterfield A.A., Hughes J., Kosterlitz H.W. (1977) Endogenous opioid peptides – multiple agonists and receptors. *Nature*;267:495–499.
2. Snyder S.H., Pasternak G.W. (2003) Historical review: opioid receptors. *TIPS*;24:198–205.
3. Mitchell S.A., Pratt M.R., Hruby V.J., Polt R. (2001) Solid-phase synthesis of O-linked glycopeptide analogues of enkephalin. *J Org Chem*;66:2327–2342 (vidi penitus).
4. Schiller P.W., Nguyen T.M.D., Weltrowska G., Wilkes B.C., Marsden B.J., Lemieux C., Chung N.N. (1992) Differential stereochemical requirements of mu vs delta-opioid receptors for ligand-binding and signal transduction – development of a class of potent and highly delta-selective peptide antagonists. *Proc Natl Acad Sci U S A*;89:11871–11875.
5. Greene D.L., Hau V.S., Abbruscato T.J., Bartosz H., Misicka A., Lipkowski A.W., Hom S., Gillespie T.J., Hruby V.J., Davis T.P.

- (1996) Enkephalin analog prodrugs: assessment of in vitro conversion enzyme cleavage characterization and blood-brain barrier permeability. *J Pharm Exp Ther*;277:1366–1375.
6. Begley D.J. (1996) The blood-brain barrier: principles for targeting peptides and drugs to the central nervous system. *J Pharm Pharmacol*;48:136–146.
 7. Watkins K.J. (2001) Peptides: a boom in the making. *Chem Eng News*;Jan. 8 issue:11–15.
 8. Hawkins B.T., Davis T.P. (2006) The blood-brain barrier/neurovascular unit in health and disease. *Pharm Rev*;57:173–185.
 9. Owens J., Lipinski C. (2003) Chris Lipinski discusses life and chemistry after the rule of five. *Drug Discov Today*;8:12–16.
 10. Egleton R.D., Bilsky E.J., Tollin G., Muthu D., John Lowery Alves I., Davis P., Porreca F., Yamamura H.I., Yeomans L., Keyari C.M., Polt R. (2005) Biosian glycopeptides penetrate the blood–brain barrier. *Tetrahedron Asymmetry*;16:65–75.
 11. Muthu D., Polt R. (2005) New prospects for glycopeptide based analgesia: glycoside-induced penetration of the blood-brain barrier. *Curr Drug Deliv*;2:59–73.
 12. Deaton K.R., Feyen E.A., Nkulabi H.J., Morris K.F. (2001) Pulsed-field gradient NMR study of sodium dodecyl sulfate micelle–peptide association. *Magn Reson Chem*;39:276–282.
 13. Chatterjee C., Majumder B., Mukhopadhyay C. (2004) Pulsed-field gradient and saturation transfer difference NMR study of enkephalins in the ganglioside GM₁ micelle. *J Phys Chem B*;108:7430–7436.
 14. Deguchi Y., Miyakawa Y., Sakurada S., Naito Y., Morimoto K., Ohtsuki S., Hosoya K., Terasaki T. (2003) Blood-brain barrier transport of a novel μ_1 -specific opioid peptide, H-Tyr-D-Arg-Phe- β -Ala-OH (TAPA). *J Neurochem*;84:1154–1161.
 15. Handa B.K., Land A.C., Lord J.A., Morgan B.A., Lance M.J., Smith C.F. (1981) Analogues of beta-LPH61–64 possessing selective agonist activity at mu-opiate receptors. *Eur J Pharmacol*;70:531–540.
 16. Sargent D.F., Schwyzer R. (1986) Membrane lipid phase as catalyst for peptide receptor interactions. *Proc Natl Acad Sci U S A*;83:5774–5778.
 17. Tallarida R.J., Murray R.B. (1987) *Manual of Pharmacological Calculations with Computer Programs*. Springer-Verlag New York Inc., New York.
 18. Egleton R.D., Mitchell S.A., Huber J.D., Palian M.M., Polt R., Davis T.P. (2001) Improved blood-brain barrier penetration and enhanced analgesia of an opioid peptide by glycosylation. *J Pharm Exp Ther*;299:967–972.
 19. Castano M.A.R.B., Fernandes M.X. (2006) Lipid membrane-induced optimization for ligand-receptor docking: recent tools and insights for the 'membrane catalysis' model. *Eur Biophys J*;35:92–103.
 20. Buchwald P., Bodor N. (1998) Octanol-water partition of nonzwitterionic peptides: predictive power of a molecular size-based model. *Proteins: Struct, Funct, Genet*;30:86–99.
 21. Kelder J., Grootenhuis P.D.J., Bayada D.M., Delbressine L.P.C., Ploemen J.-P. (1999) Polar molecular surface as a dominating determinant for oral absorption and brain penetration of drugs. *Pharm Res*;16:1514–1519.
 22. Kobata A., Yamashita K., Takasaki S. (1987) BioGel P-4 column chromatography of oligosaccharides: effective size of oligosaccharides expressed in glucose units. *Methods Enzymol*;138:84–94.
 23. Guile G.R., Rudd P.M., Wing D.R., Prime S.B., Dwek R.A. (1996) A rapid high-resolution high-performance liquid chromatographic method for separating glycan mixtures and analyzing oligosaccharide profiles. *Anal Biochem*;240:210–226.
 24. Segrest J.P., Jones M.K., Mishra V.K. (2002) Experimental and computational studies of the interactions of amphipathic peptides with lipid surfaces. *Curr Top Membr*;52:397–435.
 25. Martin I. (2004) Prediction of blood-brain barrier penetration: are we missing the point? *Drug Discov Today*;9:161–162.