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TITLE PAGE

Improved Brain Uptake and Pharmacological Activity of Dalargin Using a Peptide-Vector-Mediated Strategy

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d) **ABBREVIATIONS**

BBB: Blood-Brain Barrier; CNS: Central Nervous System; DIEA:

Diisopropylethylamine; DMF: Dimethylformamide; MPE : Maximum Possible Effect;

P-gp: P-glycoprotein; TFA: Trifluoroacetic acid.

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ABSTRACT

The blood-brain barrier restricts the passage of substances into the brain. Neuropeptides, such as enkephalins, cannot be delivered into the brain when given systemically because of this barrier. Therefore, there is a need to develop efficient transport systems to deliver these drugs to the brain. Recently, we have demonstrated that conjugation of doxorubicin or penicillin to peptide vectors significantly enhances their brain uptake. In this study, we have conjugated the enkephalin analog dalargin with two different peptide vectors, SynB1 and SynB3, in order to improve its brain delivery and its pharmacological effect. We show by *in situ* brain perfusion that vectorisation markedly enhances the brain uptake of dalargin. We also show using the hot plate model that this enhancement in brain uptake results in a significant improvement in the observed anti-nociceptive effect of dalargin. These results support the usefulness of peptide-mediated strategies for improving the availability and efficacy of central nervous system drugs.

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Brain delivery is one of the major challenges for the neuropharmaceutical industry since increasing number of hydrophilic therapeutic agents, such as anticancer drugs, antibiotics and antiviral drugs are unable to cross the blood-brain barrier (BBB). The BBB represents a complex endothelial interface in vertebrates that separates the blood compartment from the extracellular fluid compartment of the brain parenchyma. The capillaries in the brain parenchyma possess a high electrical resistance due to tight junctions between the endothelial cells, and also lack pores. Thus, the brain capillary endothelium behaves like a continuous lipid bilayer and diffusion through this BBB layer is largely dependent on the lipid solubility of the drug. Because peptides are hydrophilic, biologically unstable, and large molecules, it is difficult for them to penetrate the BBB. Even though their brain uptake is not so high, some peptides and proteins are delivered into the brain by carrier-mediated transport, receptor-mediated transport, or adsorptive-mediated transport mechanisms. One of the problems associated with the inability of many peptides and proteins to accumulate in the brain in therapeutically meaningful amounts is the efflux transport systems. For example, it has been shown that the selective delta-opioid receptors against [D-penicillamine]enkephalin (DPDPE) has a poor BBB permeability that is explained in part by P-gp mediated efflux and DPDPE is also a substrate of the rat organic anion transporting polypeptide 2 (OATP2) and human OATP-A (Gas et al, 2000; Kakyo et al, 1999).

To overcome the limited access of drugs to the brain, various strategies have been applied to direct central nervous system (CNS) drugs into the brain (Temsamani et al., 2000). Most of these methods are invasive, such as for example, surgical implantation of an intraventricular catheter followed by drug infusion into the ventricular compartment, or transient opening of the tight junctions by the intracarotid infusion of a hypertonic solution (Chamberlain et al,1993; Kroll and Neuwelt, 1998; Temsamani et al, 2000), or intracarotid

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arterial infusion of vasoactive substances such as bradykinin or bradykinin analogs (Bartus et al., 1996).

Alternative, non-invasive methods that exploit the formation of chimeric peptide or protein-drug conjugates as carriers, have also been developed. One such method relies on the presence of specific receptor-mediated transport systems in the BBB, such as for example insulin and transferrin - coupling of a non-transportable drug (peptide or protein) to an anti-receptor antibody, or other receptor-specific molecule, results in a chimeric construct that can undergo receptor-mediated transcytosis. (Bickel et al., 1993; Pardridge et al, 1994). Drug carriers such as liposomes (Zhou and Huang, 1992) and nanoparticles (Borchardt et al, 1994; Kreuter et al, 1995) have also been used for brain delivery. Despite these developments, there is still a need to develop non-invasive methods which promote the passage of inherently non-penetrating drugs through the intact brain blood vessel endothelium.

Recently, we have shown that small peptide-vectors, derived from natural peptides called protegrins, can be used to enhance brain uptake of doxorubicin and penicillin (Rousselle *et al.*, 2000, 2001, 2002). The potential of this approach as an effective delivery system for transporting drugs across the blood-brain barrier has been demonstrated in a number of animal models. The results obtained in these studies indicate that the use of peptide vectors can enhance significantly the brain uptake of doxorubicin without opening the tight junctions (Rousselle et al, 2000). The mechanism by which this vectorised doxorubicin crosses into the brain has been shown to be an adsorptive-mediated endocytosis process (Rousselle et al, 2001).

In order to assess the broad potential of this approach, we have coupled dalargin with SynB vectors and measured its brain uptake and pharmacological effect. Dalargin is a hexapeptide analog of leu-enkephalin containing D-Ala in the second position and an additional C-terminal arginine. These modifications modulate the stability of dalargin in the

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blood stream and brain while at the same time modifying to some extent its receptor selectivity. While the intracerebroventricular injection of this peptide has been shown to induce analgesic action, its systemic administration shows no activity in central analgesic mechanisms (Kalenikova et al., 1986). The reason for this is because dalargin is known not to cross the BBB.

We show in this study that SynB vectors improve the delivery of dalargin into the brain and that this enhancement in uptake is accompanied by a significant increase in its pharmacological potency in an animal model of nociception. These results support the usefulness of peptide-mediated strategies for improving the availability and efficacy of CNS drugs.

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MATERIALS AND METHODS

Animals

Adult OF1 mice (30-40g, 6-8 weeks old) were obtained from Iffa-Credo (L'Arbresle, France). Animals were maintained under standard conditions of temperature and lighting and had free access to food and water. The research adhered to the ethical rules of the French Ministry of Agriculture for experimentation with laboratory animals (Law N° 87-848).

Preparation and Characterization of Peptide Conjugates

Peptide Synthesis

The peptides were assembled by conventional solid phase chemistry using a 9-fluorenylmethoxycarbonyl/tertioButyl protection scheme (Atherton and Sheppard, 1989) and purified on preparative C18 reverse phase HPLC after trifluoroacetic acid (TFA) cleavage/deprotection. Purity of the lyophilized products was assessed by C18 reverse phase analytical HPLC and their molecular weight checked by Matrix-Assisted Laser Desorption-Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF). The peptides sequences were SynB1 (H-RGGRLSYSRRRFSTSTGR-NH₂, 2099 Da), SynB3 (H-RRLSYSRRRF-NH₂, 1395 Da) and D-SynB3 (H-rrlsysrrrf-NH₂, 1395 Da). All SynB vectors peptides were assembled on a carboxamide resin. The reference substance Dal-OH (YaGFL) was purchased from Neosystem (Strasbourg, France). Its purity and molecular weight were assessed by HPLC and MALDI-TOF respectively.

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Dal-SS-SynB Synthesis

The C-terminal cysteamide-modified dalargin was conjugated to SynB vectors activated by SPDP (3-(2-pyridyldithio)-propionic acid) by incubation of both peptides in Dimethyl Formamide (DMF) in the presence of Diisopropylethylamine (DIEA). This provided a linker containing a disulphide bond cleavable upon reduction after BBB crossing (Pardridge et al., 1994; Letvin et al., 1986). These constructs were designed to release dalargin with a C-terminal cysteamide group.

Radiolabeling of dalargin and Dal-SS-SynB

In order to introduce a radiolabel, we acetylated the N-terminal of Dal-OH, Dal-SS-SynB3, and Dal-SS-SynB1 with [¹⁴C]Acetic anhydride (Amersham, Les Ulis, France). The acetylation were performed in DMF, in the presence of DIEA. After ether precipitation, the acetylated peptides were purified on a reverse-phase semi-preparative HPLC, and lyophilized. Purity and molecular weight were checked by HPLC and MALDI-TOF respectively. The specific activity of all the compounds was 55 mCi/mmol.

Receptor binding assay

Radio-receptor assays were carried out in which competition between labeled opioid ligands and the test compound was measured using an opioid receptor-containing membrane preparation, under equilibrium conditions at neutral pH. Radioligands ([³H]DAGO, [³H]DADL, [³H]DPDPE and [³H]DSLET were purchased from New England Nuclear Corp

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(Boston, MA). Fresh calf brains were obtained locally, dissected into the appropriate brain region and homogenized in 50 volumes of Tris buffer (50 mM, pH = 7.6 at 25°C) with phenylmethyl sulfonyl fluoride (0.1 mM), EDTA (1 mM), and NaCl (100 mM), centrifuged (49000 x g for 40 min), resuspended in 0.3 M sucrose, and frozen. Tissue prepared in this manner and kept frozen at -70°C retained its binding for at least 3-4 weeks. Frozen guinea pig brains were obtained from Charles River (Wilmington, MA). The brains were thawed and the cerebella prepared and frozen as described above.

Membranes were incubated in 50 mM potassium phosphate buffer (pH=7.0 with MgSO₄ 5 mM) at 25 °C for 150 min with radioligand and various concentrations of tested compound to give a total assay volume of 2 ml. The reaction was terminated by rapid filtration over glass fibre filters. Non-specific binding was determined with levallorphan (1 μM). Receptor μ binding assays were performed using calf thalamus membranes with either [³H]DADLE (0.7 nM) in the presence of DPDPE (10 nM) for μ₁ binding or [³H]DAMGO (1 nM) in the presence of DESLET (5 nM) for μ₂ binding. MgCl₂ (5 mM) was added to the buffer in order to increase levels of specific μ binding (Clark et al., 1988). For δ binding, calf frontal cortex membranes were used with [³H]DPDPE (1 nM).

All determinations were performed in triplicate. K_i values and Hill coefficients were determined using GraphPad Prism, (San Diego, CA).

In situ mouse brain perfusion study

Surgical procedure

The uptake of free or vectorised [¹⁴C]dalargin to the luminal side of mouse brain capillaries was measured using the *in situ* brain perfusion method previously adapted in our laboratory for the study of drug uptake in the mouse brain (Dagenais *et al.*, 2000). Briefly, the right

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common carotid of ketamine/xylazine (140/8 mg/kg, ip) anesthetized mice was exposed and ligated at the heart side. The external carotid artery was ligated at the level of the bifurcation of the common carotid, rostral to the occipital artery. The common carotid was then catheterized rostrally with polyethylene tubing (0.30 mm i.d. x 0.70 mm o.d., Biotrol Diagnostic, Chennevrières-les-Louvres, France) filled with heparin (25 U/mL) and mounted on a 26G needle. The syringe containing the perfusion fluid was placed in an infusion pump (Harvard pump PHD 2000; Harvard Apparatus, Holliston, MA) and connected to the catheter. Immediately prior to the perfusion, the heart was stopped by severing the ventricles to eliminate contralateral blood flow contribution. Brains were perfused for 120 sec at a flow rate of 2.5 mL/min. At the end of the perfusion time, the mouse was decapitated and the brain removed. The right hemisphere and samples of perfusion fluid were placed in pre-weighted scintillation vials and weighted. Brain and perfusion samples were then digested for 2 hours in 1 ml of Solvable (Packard, Rungis, France) at 50°C and mixed with 9 ml of Ultima Gold XR scintillation cocktail (Packard). Total [^{14}C] and [^3H] were determined simultaneously in a Packard Tri-Carb Model 1900 TR Liquid Scintillation Analyser and activities were converted from counts per minute to disintegration per minute (dpm) with the use of internally stored quenching curves.

Brain uptake of free and vectorised [^{14}C]dalargin

The perfusate consisted of a Krebs-bicarbonate buffer, in mM : 128 NaCl, 24 NaHCO₃, 4.2 KCl, 2.4 NaH₂PO₄, 1.5 CaCl₂, 0.22 MgSO₄ and 9 D-glucose added before infusion. The solution was gassed with 95% O₂ and 5% CO₂ for pH control (=7.4) and warmed at 37°C in a water bath. Tracers were added to perfusate at concentrations of 0.4 μCi/ml for free

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dalargin, 0.1 $\mu\text{Ci/ml}$ for vectorised dalargin and 0.3 $\mu\text{Ci/ml}$ for [^3H]sucrose, the latter being a vascular marker with poor penetration of the BBB.

Determination of BBB transport constants

Briefly, calculations were carried out as previously described by Smith (1996).

The integrity of the BBB was determined in each animal by the brain vascular volume (V_v , $\mu\text{L}\cdot\text{g}^{-1}$) estimated by the tissue distribution of [^3H]sucrose from the following relationship:

$$V_v = Q_{\text{tot}}^* / C_{\text{pf}}^* \quad (1)$$

Where Q_{tot}^* is the amount of radiolabeled sucrose in the right brain hemisphere ($\text{dpm}\cdot\text{g}^{-1}$) and C_{pf}^* is the perfusate concentration of sucrose ($\text{dpm}\cdot\mu\text{l}^{-1}$).

Dalargin uptake was expressed as the volume of distribution (V_d) from the following relationships

$$V_d = Q_{\text{br}}^* / C_{\text{pf}}^* \quad (2)$$

Where Q_{br}^* is the calculated quantity of [^{14}C]tracer per gram of right brain hemisphere and C_{pf}^* ($\text{dpm}\cdot\mu\text{l}^{-1}$) is the labeled tracer concentration measured in the perfusate.

Measurement of the Antinociceptive Effect

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Antinociception was assessed in mice by the hot-plate assay. The hot-plate response has been proposed to require the activation of suraspinal mechanisms to inhibit a behavioral response (Yaksh. and Rudy, 1978).

In the hot-plate assay, mice were placed on a 54°C surface (Harvard Apparatus, Holliston, MA) and the time to lick one of the paws or escape jump was recorded as the response latency. Pre-dosing latency was determined before administration of the compounds and was 4.6 ± 1.6 sec. The hot plate latency was determined 5, 10, 15, 30 , and 45 min after intravenous injection of free or conjugated dalargin at a dose of 2 mg/kg. eq (mg base of dalargin). A maximal cutoff time of the heat was 30 sec to prevent tissue damage. To correct for individual differences in base-line latencies, the antinociceptive data (latencies) were converted to percentage maximum possible effect (% MPE) using the following formula (Brady and Holtzman, 1982).

$$\% \text{ MPE} = \frac{(\text{Postdrug latency}) - (\text{Predrug latency})}{(\text{Maximum latency}) - (\text{Predrug latency})} \times 100$$

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RESULTS

Receptor binding assay

First we determined the opioid receptor selectivities of free and vectorised dalargin using radioligand binding methods (Table 1). Dal-OH binds with low nanomolar affinity to μ - and δ - opioid receptors with about an 8-fold selectivity for μ over δ receptors. The vectorised conjugate Dal-SS-SynB3 shows a receptor selectivity and affinity similar to Dal-OH.

BBB permeability

We measured the brain uptake of free and vectorised dalargin using the in situ brain perfusion in mice. To assess the integrity of the BBB, [3 H]-sucrose was used as a marker of brain vascular volume since it does not measurably penetrate the BBB during brief (e.g. 60-120 sec) periods of perfusion. When free or conjugated dalargin were perfused, the distribution volume of [3 H]-sucrose into the right cerebral hemisphere was about 16 μ l/g indicating that the permeability of the BBB has not been altered (Figure 2). This is similar to the vascular volume values previously measured in our laboratory which is typically about 20 μ l/g. (Dagenais et al., 2000).

BBB permeabilities of free and vectorised dalargin were then assessed (Figure 3). The brain uptake of free dalargin was very low after 120 sec of perfusion ($V_d = 16.7 \pm 1.2$ μ l/g) which is comparable to the distribution volume of the [3 H]-sucrose. This perfusion time (120 sec) was chosen because it is short enough to limit risks of drug metabolism or efflux from brain to blood but high enough to measure reasonable quantities of radio-labeled dalargin in brain tissues compared with the background noise of the detection method.

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Interestingly, conjugation of dalargin to SynB1 and SynB3 via a disulfide linker (Dal-SS-SynB1 and Dal-SS-SynB3) significantly enhanced its brain uptake. The distribution volume of dalargin measured for both vectors were similar (309 +/- 82.7 for Dal-SS-SynB1 and 240 +/- 44.9 μ l /g for Dal-SS-SynB3).

In vivo analgesic studies

Free or conjugated dalargin were administered intravenously (iv) to mice and anti-nociception was determined using the Hot plate test, an assay known to be mediated by central receptors. This test measures the amount of time required for mice to react to standardized noxious stimuli. Substances which increase the reaction time are described as displaying anti-nociceptive effects, which may be interpreted as a measure of analgesia.

The results show that iv administration of free dalargin to mice at 2 mg/kg in physiological saline exhibited only a small but non-significant analgesic response (Figure 4). In contrast, conjugation of dalargin to SynB1 or SynB3 led to a considerable enhancement of analgesic activity immediately (within 5 min, the first time point) after the iv injection. Administration of the SynB1 vector alone did not produce any analgesic effect (data not shown). In order to determine if the stability of the peptide might enhance the pharmacological effect of dalargin, we have coupled it using a D-SynB3 vector. The D-form of the peptide (D-SynB3) has been shown to be more stable in serum than the L-form (SynB3) but displays a similar brain uptake (Rousselle et al, 2001). Figure 4 shows that dalargin coupled to the D-form has a similar analgesic effect as the L-form, indicating that enhancing the stability of the vector does not result in an enhancement of the analgesic effect. However, one cannot rule out that the D-form displays a different receptor binding profile.

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DISCUSSION

Neuropeptidic drugs hold great promise for the treatment of a wide variety of brain disorders such as ischemia, inflammatory and non-inflammatory neurodegenerative disorders, as well as acute, chronic or neuropathic pain syndromes. However, it is widely acknowledged that neuropeptides typically fail to reach their target after systemic administration, due to their poor transfer through the BBB. Should a strategy of penetration through the BBB be developed, the development of peptides or their synthetic analogues as neuroactive drugs would become widely used .

Here, we report the application of a peptide-mediated strategy for increasing the BBB permeability of poorly available drugs. The SynB peptides (18 amino acids for SynB1 and 10 for SynB3) translocate through biological membranes with high efficiency and have provided the basis for the development of new peptide-conjugated drugs for brain disorders. SynB vectors are derived from natural peptides called protegrins (Harwig et al., 1995). In their native form protegrins adopt antiparallel β -hairpin structures, constrained by two disulfide bridges (Aumelas et al., 1996). Replacement of the four cysteines by serines leads to linear peptides (SynB vectors) that retain their ability to cross cell membranes but which have lost their cytolytic effects. We have used these vectors as a starting point for developing new effective strategies for drug delivery into the brain (Rousselle et al., 2000; 2001). We have reported recently that vectorisation of doxorubicin and penicillin with SynB vectors enhances their brain uptake without compromising the tight junction integrity (Rousselle et al., 2000, 2002). In the present study, our rationale was to attach dalargin to SynB peptides as a vehicle for delivery of dalargin to the sites of endogenous opioid receptors in the brain. Dalargin was conjugated to the SynB vectors via a linker containing a disulfide bond. The disulfide-based

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linker system has been shown to be stable in plasma for several hours though labile in brain (Letvin et al., 1986).

The results obtained in our study indicate that SynB vectors are able to increase the threshold in nociceptive assays involving acute stimuli in mice, such as the hotplate model. This model has been interpreted to require the activation of supraspinal mechanisms to inhibit a behavioral response. This concludes that the analgesic effects we have observed are probably mediated by central mechanisms supported by the observation that, using in situ brain perfusion, dalargin conjugates are able to enter into the brain while free Dal-OH is not. In addition, we have shown that vectorised dalargin is able to bind to μ opiate receptors. The enhancement in the analgesic effect was significant for about 30 min. At later time-points, the activity of vectorised dalargin return to base-line. Interestingly, Schroeder et al. (1998) using the nanoparticle strategy have observed the same kinetics of analgesia for dalargin.

Luminal efflux transporters such as P-glycoprotein (P-gp) may restrict further BBB transport. Dalargin is a hexapeptide (molecular weight 726 Da) that is much more hydrophilic than the typical brain-penetrating drug (e.g. morphine). It has already been shown for other enkephalin analogs, such as [D-penicillamine^{2,5}]-enkephalin (DPDPE), that poor BBB permeability may in part be explained by P-gp mediated efflux (Dagenais et al., 2001). Thus, this or related efflux pumps may be responsible for the low brain uptake of dalargin. It will be interesting to see if vectorisation of dalargin will allow it to escape P-gp efflux since we have shown that doxorubicin, a P-gp substrate, bypasses the P-gp when conjugated to SynB vectors (Mazel et al., 2001).

The mechanism whereby dalargin conjugates cross the BBB is not yet clear. In general, peptides produce their central effects in brain by (i) crossing the capillary endothelial forming the BBB by either a passive diffusion or by a specific receptor-mediated mechanism, (ii) penetrating the fenestrated capillaries of the circumventricular organs (Begley, 1994) or

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(iii) undergoing endothelial uptake by phagocytosis. In contrast to these mechanisms, we have recently shown that doxorubicin vectorised with SynB1 and related vectors enters the brain by a mechanism involving adsorptive-mediated endocytosis (Rousselle et al., 2001). Three lines of evidence support this. First, the transport of vectorised doxorubicin is a saturable mechanism and the observed K_m values in the micromolar range are comparable to those found for other substrates (e.g. ebitatide, Terasaki et al., 1992; bovine serum albumine, Kumagai et al., 1987) reported to be taken up into brain via adsorptive-mediated endocytosis. Second, the brain transport does not involve a chiral receptor since no difference in brain uptake can be seen between doxorubicin coupled to SynB vectors whose amino acids are in either the L- or the D-enantiomeric form (Rousselle et al, 2001). Finally the strongest argument in favor of a mechanism involving adsorptive-mediated endocytosis is that we have reported that the passage of SynB-conjugated drugs can be inhibited in a competitive manner by polycationic molecules such as poly(L-lysine) or protamine which act as endocytosis inhibitors (Rousselle et al., 2001). The SynB-peptides used in this study are positively charged (five positive charges for SynB3) and this net positive charge is likely to play a major role in electrostatic interactions between the positive charges of the peptide vectors and the negative surface charges of the endothelial cells composing the BBB (Nagy et al., 1998). This kind of electrostatic interactions between cationic compound and negative charges suggest that the crossing of BBB by SynB vectors is via an energy dependant adsorptive-mediated endocytosis mechanisms as it was observed for other cationic peptides as ebitatide (Terasaki et al., 1992).

Other approaches for enhancing the brain uptake of dalargin into the brain have been described. For example, Kreuter et al., (1995) used a nanoparticle system for drug loading which was able to cross the BBB after adsorption and coating with polysorbate 80. A similar nanoparticle system using polysorbate 85 was described by Schroder et al. (1996). Dalargin-

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loaded nanoparticles have been shown to induce a central analgesic effect after either iv or oral administration. However the mechanism by which these complex nanoparticles cross the BBB and exhibit their effects has not been elucidated. Some authors have suggested that the antinociceptive effect of dalargin mixed with polybutylcyanoacrylate nanoparticles may originate, at least in part, from the toxicity of the carrier on the BBB and consequent opening of the tight junctions (Olivier et al., 1999). Although polysorbate 80-coated polybutylcyanoacrylate nanoparticles may be a useful experimental tool, potential therapeutic applications may be limited by the high systemic nanoparticle concentration necessary to deliver drugs to the CNS and the ensuing toxicity.

Our results show that vectorisation of dalargin enhances its brain delivery. This enhancement in brain uptake results in a significant improvement in the analgesic activity of dalargin. Finally, this study support the usefulness of peptide-mediated strategies for improving the availability and efficacy of central nervous system drugs.

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LEGENDS FOR FIGURES

Figure 1: Structure of Dal-SS-SynB

Figure 2: Distribution volume (V_d) of [^3H]-sucrose after 120 sec of perfusion of [^{14}C]Dal, [^{14}C]Dal-SS-SynB1, and [^{14}C]Dal-SS-SynB3 uptake in right hemisphere of mouse brain after 120 sec perfusion with buffer. Values are means \pm SEM (n= 4-6 mice).

Figure 3: Distribution volume (V_d) for [^{14}C]Dal, [^{14}C]Dal-SS-SynB1, and [^{14}C]Dal-SS-SynB3 uptake in right hemisphere of mouse brain after 120 sec perfusion with buffer. Values are means \pm SEM (n= 4-6 mice). **p<0. 01 versus free Dal. *p<0.05 versus free Dal.

Figure 4: Analgesic activity after intravenous injection in mice of Dal-OH, Dal-SS-SynB1, Dal-SS-SynB3, and Dal-SS-D-SynB3. Values are means \pm SEM (n=15 mice).

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Table 1: Opioid receptors binding activity in vitro. K_i values for free or coupled dalargin binding as determined by competition binding assays.

	$\mu 1$	$\mu 2$	δ
	K_i (nM)		
Dal-OH	0.41	0.63	4.79
Dal-SS-SynB3	0.33	0.87	5.91

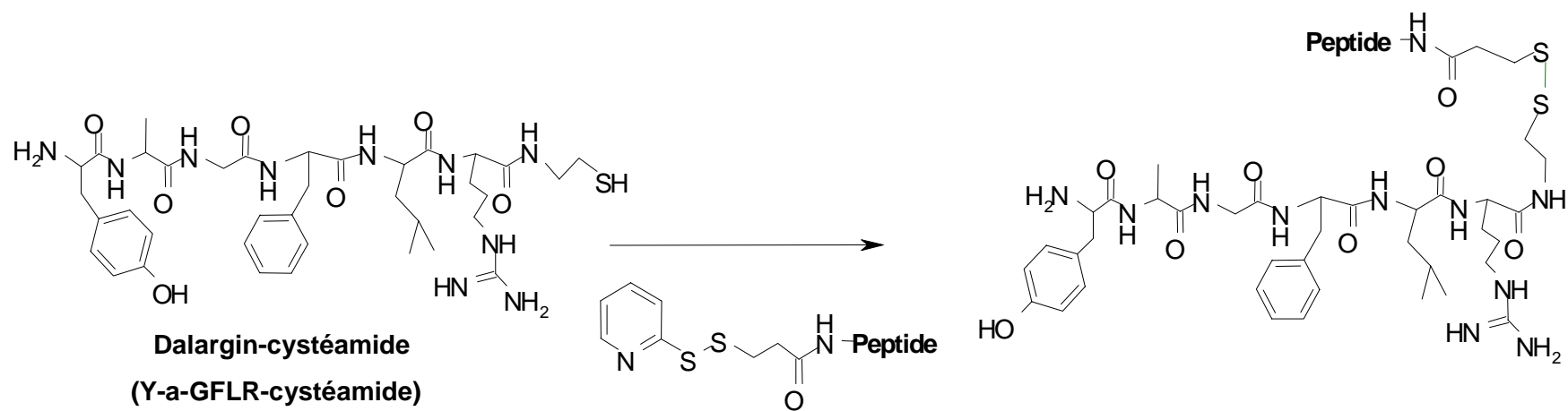


Figure 1

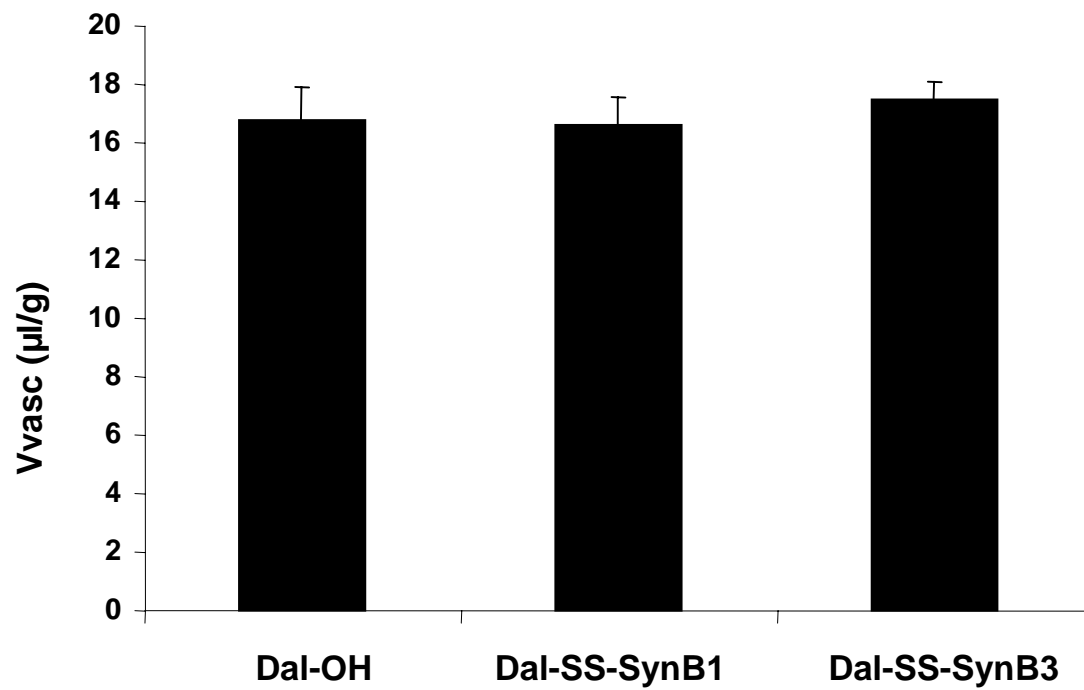


Figure 2

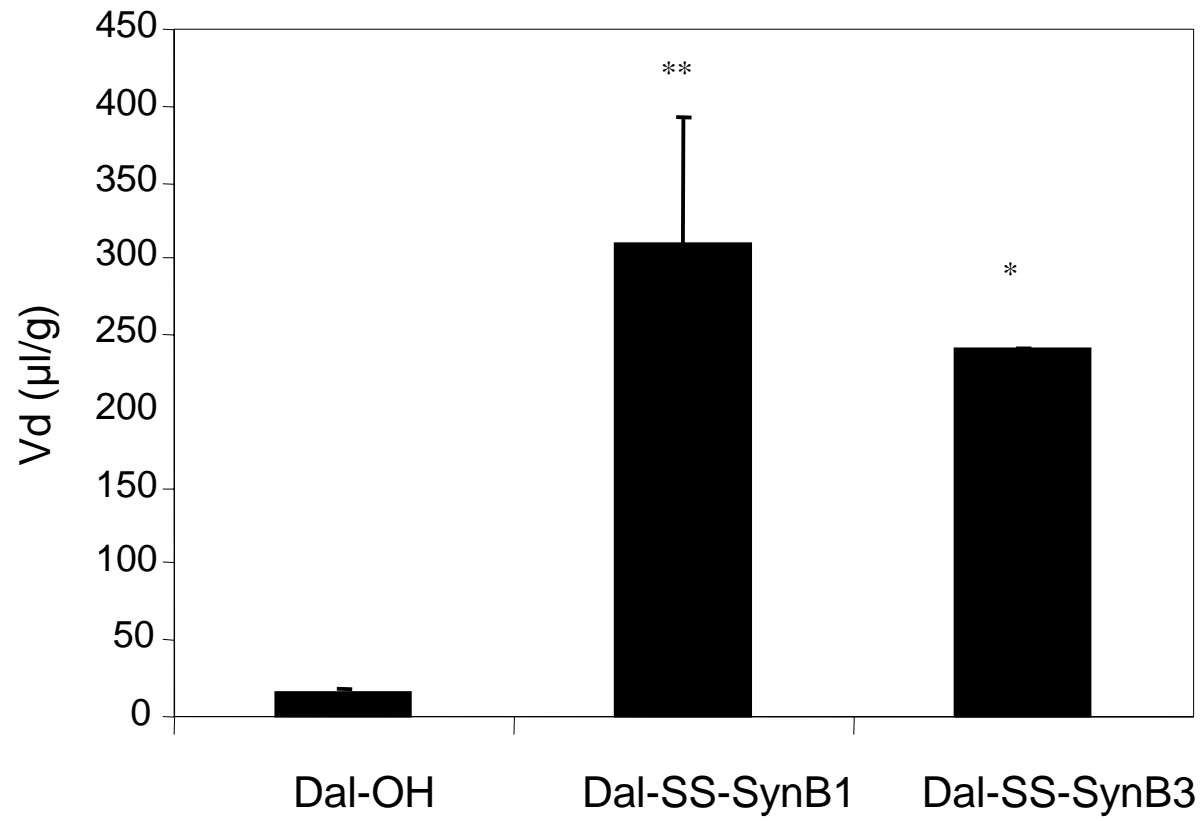


Figure 3

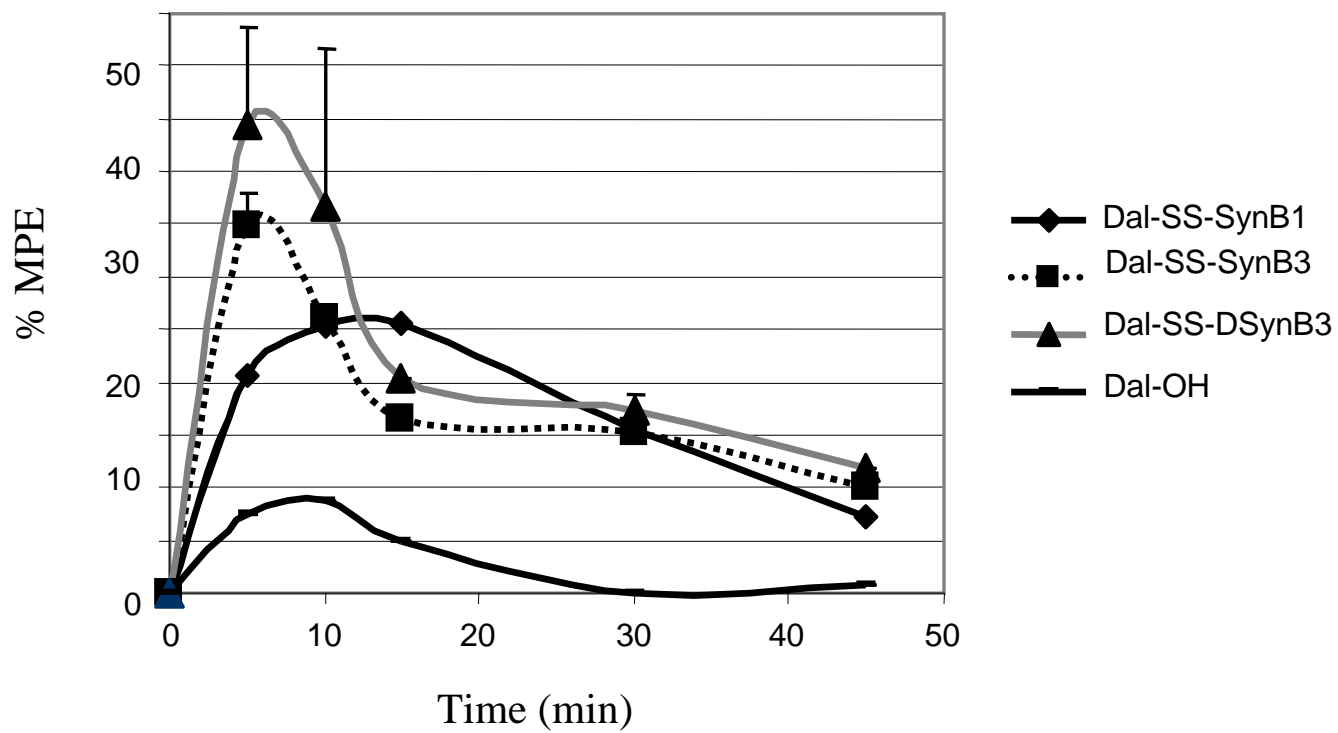


Figure 4