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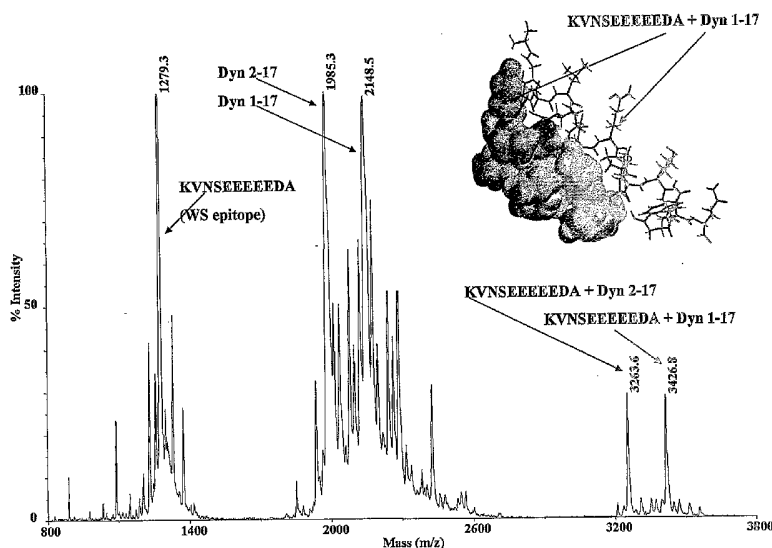
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(54) Title: IDENTIFICATION AND TARGETING OF CONSENSUS SITES BY WHICH DYNORPHIN EXERTS ITS NEUROTOXIC EFFECTS ENABLES ANALGESIA WITHOUT NEUROTOXICITY



(57) Abstract: Molecular decoyants are provided for the treatment of an organism which has been subjected to a dynorphin which exerts a neurotoxic effect only after first binding to an endogenous NMDA receptor and which exerts an analgesic effect only after first binding to an endogenous opioid receptor, the treatment being for the reduction of the neurotoxic effect without preventing the analgesic effect, having a chemical structure which functionally resembles the ligand binding site of the endogenous NMDA receptor for the dynorphin, and which does not functionally resemble the ligand binding site of the endogenous opioid receptor for the dynorphin.



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**IDENTIFICATION AND TARGETING OF CONSENSUS SITES BY WHICH
DYNORPHIN EXERTS ITS NEUROTOXIC EFFECTS ENABLES ANALGESIA
WITHOUT NEUROTOXICITY**

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Field of the Invention

Molecular decoyants are provided for the treatment of an organism which has been subjected to a dynorphin which exerts a neurotoxic effect only after first binding to an endogenous NMDA receptor and which exerts an analgesic effect only after first binding to an endogenous opioid receptor, the treatment being for the reduction of the neurotoxic effect without preventing the analgesic effect, having a chemical structure which functionally resembles the ligand binding site of the endogenous NMDA receptor for the dynorphin, and which does not functionally resemble the ligand binding site of the endogenous opioid receptor for the dynorphin.

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Background of the Invention

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Summary of the Invention

A preferred embodiment provides a molecular decoyant for treatment of an organism which has been subjected to a dynorphin which exerts a neurotoxic effect only after first binding to an endogenous NMDA receptor and which exerts an analgesic effect only after first binding to an endogenous opioid receptor, the treatment being for the reduction of the neurotoxic effect without preventing the analgesic effect, having a chemical structure which functionally resembles the ligand binding site of the endogenous NMDA receptor for the dynorphin, and which does not functionally resemble the ligand binding site of the endogenous opioid receptor for the dynorphin, the decoyant being a substance selected from the group consisting of: (a) a substance which is a portion of the NR1 subunit of the endogenous NMDA receptor for the dynorphin, the portion being (i) sufficiently small so as not to elicit an autoimmune response against the endogenous NMDA receptor when administered *in vivo*, and (ii) not substantially larger than the smallest size needed to retain the elements of the binding site of the endogenous NMDA receptor which are essential for retaining the ability of the endogenous NMDA receptor to selectively and specifically recognize and bind to the dynorphin with an affinity permitting the competition with binding of the dynorphin to the endogenous NMDA receptor and not having the elements of the binding site of the endogenous opioid receptor which are essential for retaining the ability of the endogenous opioid receptor to selectively and specifically recognize and bind to the dynorphin with an affinity so as not to permit a competition with binding of the dynorphin to the endogenous opioid receptor; (b) a substance having a chemical structure which substantially corresponds to that of the substance of (a), synthesized by chemical and/or recombinant DNA techniques and having the ability to selectively and specifically bind to the dynorphin without eliciting an autoimmune response against the endogenous NMDA receptor when administered *in vivo*; and (c) a substance having a chemical structure consisting essentially of the chemical structure of the substance of (a) or (b).

Brief Description of the Drawings

Figure A illustrates molecular decoyants for the treatment of an organism which has been subjected to a dynorphin which exerts a neurotoxic effect only after first binding to an endogenous NMDA receptor and which exerts an analgesic effect only after first binding to an endogenous opioid receptor, the treatment being for the reduction of the neurotoxic effect without preventing the analgesic effect, having a chemical structure which

functionally resembles the ligand binding site of the endogenous NMDA receptor for the dynorphin, and which does not functionally resemble the ligand binding site of the endogenous opioid receptor for the dynorphin.

Figure 1 illustrates a mass spectrum of a solution containing an equimolar mixture of Dynorphin (1-17) ($MH^+ = 2148.5$), Dynorphin (2-17) ($MH^+ = 1985.3$), KVNSEEEEEEDA peptide ($MH^+ = 1279.3$) and noncovalent complexes of Dynorphin (1-17) and KVNSEEEEEEDA peptide ($MH^+ = 3426.8$) and Dynorphin (2-17) and KVNSEEEEEEDA peptide ($MH^+ = 3263.6$). The insert shows a model of the interaction of Dynorphin (1-17) and the KVNSEEEEEEDA peptide.

Figure 2 illustrates that addition of either 10 μ M dynorphin (1-17) (A) or dynorphin (2-17) (B) to the bathing solution increased the amplitude of glutamate-evoked currents to $165 \pm 23\%$ of control values. The co-application of increasing concentrations of either D- or L- KVNSEEEEEEDA reduced the potentiation of glutamate currents evoked by the dynorphin peptides (C and D).

Figure 3 illustrates that D-KVNSEEEEEEDA and its analog L-KVNSEEEEEEDA attenuated Dynorphin A(2-17)- and ischemia/reperfusion-induced toxicity in primary cortical cultures and *in vivo*. (A) Dynorphin A(2-17) dose-dependently enhanced lactate dehydrogenase (LDH) levels. Addition of D-KVNSEEEEEEDA by itself or a mixture of Dynorphin A and D-KVNSEEEEEEDA induced less increase in LDH activity ($p < 0.05$). (B) Dynorphin A(2-17) dose-dependently decreased 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) optical density ($p < 0.05$). Such a response was not found in cells pretreated with a mixture of Dynorphin A and D-KVNSEEEEEEDA ($p < 0.05$). (C) Intracerebroventricular administration of D-KVNSEEEEEEDA or L-KVNSEEEEEEDA markedly reduced cortical infarction induced by middle cerebral arterial ligation. The volume of infarction was significantly decreased in animals pretreated with D-KVNSEEEEEEDA or L-KVNSEEEEEEDA, as compared to those pretreated with vehicle. The volume of infarction = (sum of the infarction area in 7 consecutive brain slices (mm^2)) $\times$ (thickness of the slice, 2 mm). (D) The area of the largest infarction in a slice from each rat was significantly diminished by KVNSEEEEEEDA analogs. (E) The total number of infarcted slices was significantly diminished by KVNSEEEEEEDA analogs. (F) Pretreatment with KVNSEEEEEEDA increases locomotor activity in stroke rats. Animals were tested 48 hours after ischemia/reperfusion. The motor activity was examined 0-30 min after placing the animals in the chamber. Horizontal activities are measured by the

sum of the total number of beam interruptions that occurred in the horizontal during the 30 min testing period. Total distance traveled and movement time were increased in the D- and L-KVNSEEEEEEDA-pretreated rats, as compared to the vehicle-treated controls. Pretreatment with D- or L-KVNSEEEEEEDA significantly reduced ischemia-induced body asymmetry as examined by an elevated body swing test (* $p < 0.05$, One way ANOVA + Newman-Keuls' test).

Figure 4. A. Neuronal survival. B. Tactile allodynia. C. Paralysis. D. Rota-Rod. All graphs show a drastic improvement in animals given the KVNSEEEEEEDA peptide in the presence of dynorphin.

Figure 5A illustrates that KVNSEEEEEEDA attenuates dynorphin-induced hind limb paralysis as calculated by a 5-point paralysis rating scale.

Figure 5B illustrates the ability of KVNSEEEEEEDA to reverse paralysis (closed diamonds) when given post paralysis. *, significant difference relative to Dyn 75 nmol (open square). ^, significant difference relative to Dyn 100 nmol.

Figure 6 illustrates that KVNSEEEEEEDA prevents the expression of antinociceptive tolerance to morphine. % MPE - 10 min after morphine i.t.

Brief Description of the Sequences

Dynorphin (1-17)	SEQ ID NO:1
NMDA receptor NR1 subunit	SEQ ID NO:2
EEEEED	594-599 of SEQ ID NO: 2
KVNSEEEEEEDA	590-600 of SEQ ID NO: 2

Detailed Description of the Preferred Embodiment

Dynorphin A and its major biotransformation product, dynorphin A(2-17), elicit a number of pathological effects including neurological dysfunction and neuronal cell death (Caudel, R.M. & Manes, A.J. 2000 *Pain* **87**:235-239). These actions are not opioid receptor-mediated but are reduced by N-methyl-D-aspartate receptor (NMDA) antagonists suggesting that the neurotoxic effects of dynorphin are NMDA receptor-mediated (Pohl, M. *et al.* 1997 *Brain Research* **749**:18-28; Herman, B.H. & Goldstein, A. 1985 *J Pharmacol Exp Ther* **232**:27-32; Caudle, R.M. & Isaac, L. 1987 *Brain Research* **435**:1-6; Bakshi, R. & Faden, A.I. 1990 *Brain Research* **507**:1-5; Caudle, R.M. & Isaac, L. 1988 *Brain Research* **443**:329-332; Hauser, K.F. *et al.* 1999 *Exp Neurol* **160**:361-375; Brauneis, U. *et al.* 1996 *J Pharmacol Exp Ther* **279**:1063-1068; Caudle, R.M. & Dubner, R. 1998 *Neuropeptides*

32:87-95). Here we report the identification of a conserved sequence of the NR1 subunit of the NMDA receptor complex that binds dynorphin peptides non-covalently. We show that synthetic peptides containing this sequence form stable complexes with dynorphin, and prevent the potentiation of NMDA receptor-activated responses produced by dynorphin A and dynorphin (2-17). These peptides prevent dynorphin-induced cell death and protect against ischemic brain injury. Furthermore, they reduce dynorphin-evoked dysesthesias and motor neuron loss in the spinal cord. These data demonstrate that peptides which complex with dynorphin and prevent its binding to the NR1 receptor subunit are neuroprotective, and, thus, they are envisioned as effective therapeutic agents for the treatment of nerve injury.

Header A.

The opioid peptide, dynorphin A, has been implicated in the pathogenesis of ischemic brain injury, and neuropathic pain. Dynorphin A-derived peptides are elevated following brain or spinal cord trauma, and sustained exposure to these peptides is neurotoxic. Although opiates are potent analgesics, the intrathecal infusion of dynorphin to experimental animals produces abnormal pain (e.g., allodynia), hindlimb paralysis and a loss of neurons in the spinal cord. These actions of dynorphin are not opioid receptor-mediated since they are insensitive to opioid receptor antagonists. Moreover, truncated peptides that lack the N-terminus essential for opioid receptor binding retain full activity. *In vitro* studies have shown that dynorphin peptides bind to the NMDA receptor and can potentiate NMDA-evoked currents in brain and heterologous expression systems. NMDA receptor antagonists reduce dynorphin-induced tactile allodynia and paralysis and prevent dynorphin-induced cell loss in cortical and spinal cord cultures. Although these findings suggest an involvement of the NMDA receptor complex in mediating both the pronociceptive and neurotoxic actions of dynorphin, the mechanism by which dynorphin and related peptides modulate NMDA receptor function is unknown. We report here that the presence of arginine 6, 7 and lysine 11, 13 in the dynorphin molecule enables dynorphin and related peptides to bind non-covalently to a conserved acidic sequence of the NR1 subunit (594-599, EEEEEED) via salt bridging and cation- π interactions. We show that synthetic peptides containing this sequence form stable complexes with dynorphin and prevent the potentiation of NMDA-mediated cation currents elicited by N-truncated dynorphin in *Xenopus* oocytes expressing NR1A/2B subunits. The administration of these peptides to rats prevents ischemic brain injury and neuronal cell death in cortical cultures

and attenuates the tactile allodynia, flaccid paralysis and loss of spinal cord motor neurons elicited by intrathecal infusion of dynorphin or N-truncated dynorphin.

Header B.

Beginning with Figure A, the novel solution to the problem of treatment of an organism which has been subjected to dynorphin which exerts a neurotoxic effect only after first binding to an endogenous NMDA receptor and which exerts an analgesic effect only after first binding to an endogenous opioid receptor, the treatment being for the reduction of the neurotoxic effect without preventing the analgesic effect, involves the identification of the molecular structure of the ligand binding site in the native receptor and the production of mimic ligand binding sites.

These mimic ligand binding sites functionally resemble the ligand binding site of the endogenous NMDA receptor for the dynorphin, and do not functionally resemble the ligand binding site of the endogenous opioid receptor for the dynorphin.

The mimic ligand binding sites of the present invention retain the elements of the binding site of the endogenous NMDA receptor which are essential for retaining the ability of the endogenous NMDA receptor to selectively and specifically recognize and bind to the dynorphin with an affinity permitting the competition with binding of the dynorphin to the endogenous NMDA receptor and not having the elements of the binding site of the endogenous opioid receptor which are essential for retaining the ability of the endogenous opioid R to selectively and specifically recognize and bind to the dynorphin with an affinity so as not to permit a competition with binding of the dynorphin to the endogenous opioid receptor. The mimic ligand binding sites thus act as decoys. Such substances have been denominated "molecular decoyants."

In one embodiment, the molecular decoyants are taken from a substance which is a portion of the NR1 subunit of the endogenous NMDA receptor for the dynorphin, the portion being sufficiently small so as not to elicit an autoimmune response against the endogenous NMDA receptor when administered *in vivo.*, and not substantially larger than the smallest size needed to retain the elements of the binding site of the endogenous NMDA receptor which are essential for retaining the ability of the endogenous NMDA receptor to selectively and specifically recognize and bind to the dynorphin with an affinity permitting the competition with binding of the dynorphin to the endogenous NMDA receptor and not having the elements of the binding site of the endogenous opioid receptor which are essential for retaining the ability of the endogenous opioid receptor to selectively

and specifically recognize and bind to the dynorphin with an affinity so as not to permit a competition with binding of the dynorphin to the endogenous opioid receptor.

In another embodiment, the molecular decoyants are taken from a substance having a chemical structure which substantially corresponds to that of the substance described
5 above, synthesized by chemical and/or recombinant DNA techniques and having the ability to selectively and specifically bind to the dynorphin without eliciting an autoimmune response against the endogenous NMDA receptor when administered *in vivo*.

In another embodiment, the molecular decoyants are taken from a substance having a chemical structure consisting essentially of the chemical structure of the substance of
10 either of the above alternatives.

It is known that natural receptors are rather large structures, comprising some hundreds of amino acids, and can be as large as a molecular weight of about 250,000. The specific binding site, however, is much smaller and this opens up the possibility of preparing artificial binding sites, which are effective, yet which comprise a much smaller
15 number of amino acids, preferably less than 100, and which have therefore a considerably lower molecular weight and thus a correspondingly lower immunogenicity. It has been found possible to prepare such binding-site-mimicking molecular decoyants which are adapted to bind specific ligands with a size on the order of about 10 amino acids. Such rather small peptide structures can be prepared by physically dividing the endogenous
20 receptor or they can be prepared synthetically by the preparative procedures of peptide chemistry, such as Merrifield synthesis, or of peptidomimetic chemistry, or by genetic engineering. This opens up the possibility of large scale production of such specific peptide structures and their use as active materials in the treatment of an organism which has been subjected to dynorphin which exerts a neurotoxic effect only after first binding to an
25 endogenous NMDA receptor and which exerts an analgesic effect only after first binding to an endogenous opioid receptor, the treatment being for the reduction of the neurotoxic effect without preventing the analgesic effect.

An embodiment provides a method for the treatment of an organism which has been subjected to a dynorphin which exerts a neurotoxic effect only after first binding to an
30 endogenous NMDA receptor and which exerts an analgesic effect only after first binding to an endogenous opioid receptor, the treatment being for the reduction of the neurotoxic effect without preventing the analgesic effect, comprising: administering to the organism, in a quantity sufficient to reduce the neurotoxic effect without preventing the analgesic

effect, a molecular decoyant having a chemical structure which functionally resembles the binding site of the endogenous NMDA receptor for the dynorphin, and which does not functionally resemble the ligand binding site of the endogenous opioid receptor for the dynorphin, the decoyant being a substance selected from the group consisting of: (a) a substance which is a portion of the NR1 subunit of the endogenous NMDA receptor for the dynorphin, the portion being (i) sufficiently small so as not to elicit an autoimmune response against the endogenous NMDA receptor when administered *in vivo*, and (ii) not substantially larger than the smallest size needed to retain the elements of the binding site of the endogenous NMDA receptor which are essential for retaining the ability of the endogenous NMDA receptor to selectively and specifically recognize and bind to the dynorphin with an affinity permitting the competition with binding of the dynorphin to the endogenous NMDA receptor and not having the elements of the binding site of the endogenous opioid receptor which are essential for retaining the ability of the endogenous opioid receptor to selectively and specifically recognize and bind to the dynorphin with an affinity so as not to permit a competition with binding of the dynorphin to the endogenous opioid receptor; (b) a substance having a chemical structure which substantially corresponds to that of the substance of (a), synthesized by chemical and/or recombinant DNA techniques and having the ability to selectively and specifically bind to the dynorphin without eliciting an autoimmune response against the endogenous NMDA receptor when administered *in vivo*; and (c) a substance having a chemical structure consisting essentially of the chemical structure of the substance of (a) or (b).

Another embodiment provides use of a molecular decoyant for the manufacture of a medicament for the treatment of an organism which has been subjected to a dynorphin which exerts a neurotoxic effect only after first binding to an endogenous NMDA receptor and which exerts an analgesic effect only after first binding to an endogenous opioid receptor, the treatment being for the reduction of the neurotoxic effect without preventing the analgesic effect, having a chemical structure which functionally resembles the ligand binding site of the endogenous NMDA receptor for the dynorphin, and which does not functionally resemble the ligand binding site of the endogenous opioid receptor for the dynorphin, the decoyant being a substance selected from the group consisting of: (a) a substance which is a portion of the NR1 subunit of the endogenous NMDA receptor for the dynorphin, the portion being (i) sufficiently small so as not to elicit an autoimmune response against the endogenous NMDA receptor when administered *in vivo*, and (ii) not

substantially larger than the smallest size needed to retain the elements of the binding site of the endogenous NMDA receptor which are essential for retaining the ability of the endogenous NMDA receptor to selectively and specifically recognize and bind to the dynorphin with an affinity permitting the competition with binding of the dynorphin to the endogenous NMDA receptor and not having the elements of the binding site of the endogenous opioid receptor which are essential for retaining the ability of the endogenous opioid receptor to selectively and specifically recognize and bind to the dynorphin with an affinity so as not to permit a competition with binding of the dynorphin to the endogenous opioid receptor; (b) a substance having a chemical structure which substantially corresponds to that of the substance of (a), synthesized by chemical and/or recombinant DNA techniques and having the ability to selectively and specifically bind to the dynorphin without eliciting an autoimmune response against the endogenous NMDA receptor when administered *in vivo*; and (c) a substance having a chemical structure consisting essentially of the chemical structure of the substance of (a) or (b).

In another embodiment, the method (or use) further comprises administering (or use of) the molecular decoy together with dynorphin to reduce the neurotoxic effect of the administered dynorphin.

In another embodiment, the method (or use) further comprises administering (or use of) the molecular decoy in the absence of dynorphin to reduce the neurotoxic effect of non-administered dynorphin.

In another embodiment, the method (or use) further comprises administering the molecular decoy (or use of the molecular decoy for administration) to a patient with stroke or spinal cord injury to reduce the neurotoxic effect of dynorphin.

In another embodiment, the method (or use) further comprises administering the molecular decoy (or use of the molecular decoy for administration) to a patient in a neuropathic pain state, such as in AIDS, to reduce the neurotoxic effect of dynorphin.

In another embodiment, the method (or use) further comprises administering the molecular decoy (or use of the molecular decoy for administration) to a patient at risk for antinociceptive tolerance to morphine and other opiates to reduce the risk, hence allowing the relief of pain with minimal opiate dosage, thus making the patient comfortable while reducing the side effects of the opiate.

As described below, KVNSEEEEEEDA has proven to be a molecular decoy for treatment of an organism which has been subjected to a dynorphin which exerts a

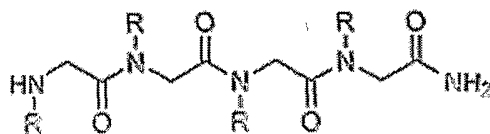
neurotoxic effect only after first binding to an endogenous NMDA receptor and which exerts an analgesic effect only after first binding to an endogenous opioid receptor, the treatment being for the reduction of the neurotoxic effect without preventing the analgesic effect, having a chemical structure which functionally resembles the binding site of the endogenous NMDA receptor for the dynorphin, and which does not functionally resemble the ligand binding site of the endogenous opioid receptor for the dynorphin, the decoyant being a substance which is a portion of the NR1 subunit of the endogenous NMDA receptor for the dynorphin.

The fact that KVNSEEEEEEDA has been proven to be a decoyant against dynorphin is only a case in point for the general claim of molecular decoyants as therapeutic agents. In the specific case of the NMDA receptor, KVNSEEEEEEDA, or improved versions of this molecular decoyant, can serve as an antidote against neurotoxic effects of dynorphin elicited through its binding to the NMDA receptor.

It should be understood that KVNSEEEEEEDA is but an intermediate tool which, by its genetic manipulation or chemical processing, would allow those of ordinary skill in the art to design even more efficient dynorphin decoyants. Thus, the present invention is intended to include not only the specific 11 amino acid sequence of KVNSEEEEEEDA, but variations and derivatives thereof which maintain, and preferably improve, its functional or pharmacological characteristics. For example, modified peptide sequences can be readily prepared and tested by routine techniques for preferred dynorphin-binding characteristics so as to more effectively compete against the NMDA receptor. Such modification may involve substitution, deletion or insertion of amino acids or their chemical modification. For example, longer lived decoyants may be obtained in this manner. As enzymatic degradation of the decoyants *in vivo* may cause some decoyants to be relatively short-lived, one method of preventing such degradation would be by making synthetic peptides containing D-amino acids. Alternatively, organic molecules, *i.e.*, not proteinaceous, can be designed so as to satisfy the physico-chemical requirements of a decoyant which must form a functional interface with the dynorphin.

One example of such a molecule is a peptidomimetic. A peptidomimetic is a compound containing non-peptidic structural elements that is capable of mimicking or antagonizing the biological action(s) of a natural parent peptide. A peptidomimetic does not have peptide bonds, so it cannot be hydrolyzed by endopeptidases or exopeptidases that cleave peptides. Peptidomimetics, however, can be designed with side chains similar to

those of peptides of interest. Hence they would be able to interact with the sites of interest on receptors, while at the same time act as inhibitors to cleaving enzymes.



As shown in this cartoon, the side chain is attached to a nitrogen atom, rather than a carbon atom, but the acetamide bond is preserved.

It should further be understood that the decoyants of the present invention can be modified by extending the polypeptide or by adding specific chemical moieties intended to aid in drug design or to permit the decoyants to be used for additional utilities. One such modification would be to extend the polypeptide by moieties intended to affect solubility, *e.g.*, by the addition of a hydrophilic residue, such as serine. Furthermore, the decoyant could be extended for the purpose of stabilization and preservation of a desired conformation, such as by adding cysteine residues for the formation of disulfide bridges.

Another reason to modify the decoyants would be to make the decoyant detectable, even after administration. This might be done by radioiodination with a radioactive iodine isotope, directly, or by adding tyrosine for subsequent radioiodination. Such detectable decoyants could be used to detect the presence and/or location of dynorphin.

A further reason for modifying decoyants would be for accelerated clearance of the conjugated dynorphin from the body. For example, a decoyant linked to an asialoglyco-moiety would be expected to be cleared by the liver. Thus, for example, a decoyant mimicking the NMDA receptor site of dynorphin and containing such an asialoglyco-moiety, or any other moiety which would aid in its clearance, could be used to inactivate and quickly remove excess dynorphin after the therapy is completed in order to reduce neurotoxic effects.

Another reason for modifying decoyants would be to increase their efficiency that allows for their use in much lower concentrations. According to the Net Phos and other phosphorylation programs the likelihood of the serine residue in KVNSEEEEEEDA being phosphorylated is 99.9 %. We have shown that the presence of a phosphate group doubles the potency of an acidic peptide. Therefore, adding a phosphate group to a molecular decoyant of the present invention is also within the scope of the invention.

Proof of the effectiveness of KVNSEEEEEEDA as a decoyant against dynorphin *in vivo* establishes the operability of the general concept of the present invention. The first

requirement of a decoyant in accordance with the present invention is that it be a mimic of the NMDA receptor, *i.e.*, it must functionally resemble the binding site, although it may differ physically. The term "functionally resemble" means that the decoyant binds to the NMDA receptor-binding site on dynorphin in a selective and specific manner and with
5 reasonable affinity. Replacing the five adjacent glutamate residues with five adjacent aspartate residues would give a peptide with identical properties to the example and thus would functionally resemble the example, so long as the residues were acidic residues.

A decoyant in accordance with the present invention should not be substantially immunogenic. Reduction of size is a means of diminishing the immunogenicity of a
10 substance, but not all large molecules are as immunogenic as some small molecules. To be classified as a decoyant in accordance with the present invention, the substance must be substantially non-immunogenic in the system of the host, regardless of the size of the substance, although the smallest possible size is preferred. It is very important, however, that the decoyant not be sufficiently immunogenic to elicit an autoimmune response against
15 the NMDA receptor when administered *in vivo*.

A decoyant in accordance with the present invention must comprise the essential elements of the binding site of NMDA receptor and not substantially more. For the purpose of this invention, the "essential elements" of a binding site are defined as those elements essential for the decoyant activity, *i.e.*, ligand recognition and binding. A receptor consists
20 of many residues, only a few of which are involved in ligand recognition and binding. However, as discussed above, the decoyants of the present invention may be further modified for purposes of drug design. Thus, for example, the entire NR1 subunit of the NMDA receptor would not qualify as a decoyant, being both immunogenic and also considerably longer than necessary. The NR1 subunit does, however, contain the
25 information needed for the design and construction of a decoyant, *i.e.*, residues 590-600. The fact that some additional peptide units may also be present, for example to improve the solubility of the essential required sequence, would not remove the structure from the category of decoyant as long as it is still substantially non-immunogenic and it is still selective, specific and of reasonable affinity. Addition of sugar molecules could be a
30 modification with the same effect. Thus, additions to the molecule for the purpose of drug design are not considered when determining whether the substance contains substantially more than the elements of the NMDA receptor which are required for binding to the dynorphin.

The second requirement of a decoyant in accordance with the present invention is that it not be a mimic of the opioid receptor, *i.e.*, it must not functionally resemble the binding site, indeed, it may differ physically. The term "not functionally resemble" means that the decoyant does not bind to the opioid receptor-binding site on dynorphin in a selective and specific manner and with reasonable affinity.

A decoyant is a drug designed to intercept an agent, *e.g.*, dynorphin, having an undesired effect. As long as dynorphin exerts its neurotoxic effect only after binding to a non-opioid, *e.g.*, NMDA receptor site, a decoyant can be designed in accordance with the present invention to prevent such binding and thereby eliminate such undesired effect.

The decoyants of the present invention may be administered to an animal, including a human patient, in order to reduce the undesired effects of the dynorphin for which it was designed. Such decoyants can be used not only for the treatment of humans, but also for the treatment of other animals, including mammals, poultry, fish, etc. The specific effective dosages for the treatment to reduce the neurotoxic effect of dynorphin without preventing its analgesic effect can readily be empirically determined by those of ordinary skill in the art without undue experimentation. However, those skilled in the art will understand that the dosage of decoyant will depend to some extent on the amount of dynorphin in the system of the host. The ratio of decoyant to dynorphin molecules is preferably in the range of 10:1 to 1:10. Preferably, the amount of dynorphin in the bloodstream or cerebrospinal fluid of the host will be monitored and the decoyant dosage adjusted accordingly during the course of treatment.

Compositions within the scope of the present invention include compositions wherein the decoyant is present in an effective amount to achieve its intended purpose. Determination of the effective amounts is within the skill in the art.

In addition to the decoyants of the present invention, the pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Preferably, the preparations, particularly those which can be administered by injection, contain from about 0.1 to 99 percent, and preferably from about 25 to 85 percent by weight, of the active ingredient, together with the excipient.

Any conventional route of administration may be used for the decoyants of the present invention. Although the preferred mode of administration is by injection, *e.g.*,

intravenously, intradermally, intraperitoneally, intramuscularly, intrathecally, etc., they may also be administered orally, by suppository or by any other route.

Other non-conventional means of administration can be envisioned which are also intended to be comprehended within the scope of the present invention. For example, bacterial expression systems are known in the art which would actually secrete a soluble form of the expressed protein into the medium, and other viral expression systems are available. It could be contemplated that such a secreting expression system could be used to generate the decoyant from within the host rather than producing it *ex vivo* and administering it to the host. Obviously, the secreting system must be compatible with the host. The term "administration" as used in the present specification and claims is intended to include such *in vivo* secretion systems.

The pharmaceutical preparations of the present invention are manufactured in a manner which is itself known, for example, by means of conventional mixing, dissolving, or lyophilizing processes. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters such as ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension such as sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. The decoyants of the present invention may also be administered in the form of liposomes, pharmaceutical compositions in which the active ingredient is contained either dispersed or variously present in corpuscles consisting of aqueous concentric layers adherent to lipidic layers. The active ingredient may be present both in the aqueous layer and in the lipidic layer, or, in any event, in the non-homogeneous system generally known as a liposomic suspension.

Header C.

Peptides or proteins containing two or more adjacent Arginines (Arg, R) form non-covalent complexes with those containing two or more adjacent glutamic (Glu, E) or aspartic acids (Asp, D) (Woods, A.S. & Huestis, M.A. 2001 *JASMS* 12:88-96; Woods, A.S. *et al.* 2002 *JASMS* 13:166-169). The side chain of Arg's functional group is a guanidinium (basic and positively charged), and that of Asp and Glu is a carboxyl group (acidic and negatively charged), thus resulting in Coulombic interaction (salt bridge formation).

Dynorphin (YGGFLRRIRPKLKWDNQ) (SEQ ID NO: 1) and N-truncated dynorphin fragments are extremely basic peptides due to the presence of five basic residues, three Arg in positions 6, 7, 9 and two lysines (Lys, K) in positions 11, 13. Examination of the sequences of cloned NMDA receptor subunits revealed that the NR1 subunit (GenBank
 5 accession No. L05666, SEQ ID NO: 2) contains five adjacent Glu followed by an Asp residue (594-599 of SEQ ID NO: 2, EEEEEED):

MSTMRLTLALLFSCSVARAACDPKIVNIGAVLSTRKHEQMFREAVNQANKRHGS
 WKIQLNATSVTHKPNAIQMALSVCEDLISSQVYAILVSHPTPNDFHTPTPVSYTAG
 FYRIPVLGLTTRMSIYSDKSIHLSFLRTVPPYSHQSSVWFEMMRVYSWNHILLVSD
 10 DHEGRAAQKRLETLLERESKAQKVLQFDPGTKNVTALLMEAKELEARVILSASE
 DDAATVYRAAAMLNMTGSGYVWLVGEREISGNALRYAPDGILGLQLINGKNESA
 HISDAVGVAQAVHELLEKENITDPPRGCVGNTNIWKTGPLFKRVLMSKYADGV
 TGRVEFNEDGDRKFANYSIMNLQNRKLVQVGIYNGTHVIPNDRKIIWPGGETEKPR
 GYQMSTRCLKIVTIHQEPFVYVKPTLSDGTCKEEFTVNGDPVKKVICTGPNDTSPGSP
 15 RHTVPQCCYGFCDLLKLARTMNFTYEVHLVADGKFGTQERVNNSNKKKEWNGM
 MGELLSGQADMIVAPLTINNERAQYIEFSKPFKYQGLTILVKKEIPRSTLDSFMQPFQ
 STLWLLVGLSVHVAVMLYLLDRFSPFGRFKVNSEEEEEEDALTSSAMWFSWGV
 LLNSGIGEGAPRSFSARILGMVWAGFAMIIVASYTANLAAFLVDRPEERITGINDPR
 LRNPDKFIYATVKQSSVDIYFRRQVELSTMYRHMEKHNYESAAEAIQAVRDNKL
 20 HAFIWDSAVLEFEASQKCDLVTTGELFFRSGFGIGMRKDSPWKQNVSLKSHEN
 GFMEDLDKTWVRYQECDSRSNAPATLTFENMAGVFMLVAGGIVAGIFLIFIEIAYK
 RHKDARRKQMQLAFAAVNVWRKNLQQYHPTDITGPLNLSDPSVSTVV.

This sequence EEEEEED is conserved in all species. We hypothesized that the adjacent guanidinium groups on Arg_{6,7} of dynorphin interact with adjacent carboxyl groups
 25 of Glu on the NR1 subunit resulting in the formation of a powerful salt bridge. To test this hypothesis we used mass-spectrometry to determine if, in solution, dynorphin (1-17) or (2-17) form a stable complex with an epitope of NR1 containing the EEEEEED sequence. Analysis of 0.3 µl of an equimolar solution of dynorphins (1-17), (2-17) and the epitope of NR1 (res. 590-600) containing the acidic residues KVNSEEEEEEDA (final concentration of
 30 222 fmol/µl of each), revealed molecular ions (MH⁺) at amu 1279.3 (NR1 res. 590-600), 1985.3 (dyn 2-17), 2148.5 (dyn 1-17), 3263.6 corresponding to a complex of dynorphin (2-17) and KVNSEEEEEEDA, and 3426.8 corresponding to a complex of dynorphin (1-17) and KVNSEEEEEEDA (Figure 1), confirming that peptides containing the epitope EEEEEED

form a non-covalent complex with both dynorphin (1-17) or (2-17). Shorter fragments of dynorphin that contain Arg 6 and 7 are also neurotoxic (Hauser, K.F. *et al.* 2001 *Exp Neurol* 168:78-87) and formed a salt bridge with the NR1 epitope KVNSEEEEEEDA. In contrast, those fragments that lack Arg 6, 7, and are not neurotoxic, did not. To test if a larger epitope of NR1 containing the KVNSEEEEEEDA epitope would form a non-covalent complex with dynorphin, a 51 amino acid epitope (res. 576-626) of the NR1 subunit was synthesized. When equimolar solutions of dynorphins (1-17) or (2-17) (666 fmol/ μ l) were added to this epitope, non-covalent complexes were observed at amu 7890 and 7726.8 amu, again confirming peptide-epitope complexing. A chymotryptic digest of dynorphin (1-17), KVNSEEEEEEDA (WS ept), and the 51 amino acid epitope of NR1 (NMDA ept) revealed the presence of 20 complexed fragments (Table 1). Importantly, all complexes consisted of dynorphin fragments containing the RR motif and KVNSEEEEEEDA or the 51 residue epitope fragments containing two or more Glu residues indicating that the non-covalent complex formed is stable and resistant to disruption by enzymatic attack. Indeed, modeling of the secondary structure of dynorphin and NR1 epitope (Pole Bio-Informatique Lyonnais: http://pbil.ibcp.fr/cgi-bin/secpred_sopma.pl) revealed that both Arg_{6, 7} and the EEEEEED epitope are located on alpha helixes, an orientation that would further facilitate salt bridge formation. In addition, the presence of three aromatic residues in dynorphin (Tyr₁, Phe₄, and Trp₁₄) favor the formation of cation- π interactions (Woods, A. *et al.* 2003, ASAP Web Release Date 25-Jan-2003, *J. Proteome Res.*, in press) with the Arg₅₈₈, Lys₅₉₀ and Asn₅₉₂ residues of the NR1 subunit, while the guanidinium in Arg₉ and the NH₂ groups in Lys_{11, 13} of dynorphin facilitate hydrogen bonding with the carboxyl groups of Glu and Asp that do not form a salt bridge with Arg_{6, 7}. Molecules involved in non-covalent interactions are in a constant flux of association and dissociation. However, in the case of dynorphin and the NR1 subunit two different types of interactions are involved: electrostatic and cation- π . The likelihood of simultaneous dissociation of two different types of interactions is remote. In addition, the Spartan program was used to calculate and model the surface potential of dynorphin and KVNSEEEEEEDA. The generated peptide models reflect the positive charges on dynorphin and the negative charges on the KVNSEEEEEEDA epitope thus supporting our finding, while emphasizing the dominance of the electrostatic interaction (Figure 1).

Table 1. Non-covalent complexes detected after overnight digest with chymotrypsin

Components	MW of Components	MH ⁺
Dyn 5-16 + WS ept 1-11	1594.9 + 1278.3	2874.2
Dyn 6-17 + WS ept 1-11	1609.9 + 1278.3	2889.2
Dyn 3-17 + WS ept 3-11	1926.1 + 1050.9	2978.0
Dyn 5-17 + WS ept 1-11	1723.1 + 1278.3	3003.4
Dyn 2-17 + WS ept 3-11	1984.3 + 1050.9	3036.2
Dyn 2-17 + WS ept 2-10	1984.3 + 1078.4	3063.7
Dyn 1-17 + WS ept 4-10	2147.5 + 936.3	3084.8
Dyn 1-16 + WS ept 2-11	2019.4 + 1150.1	3170.5
Dyn 1-17 + WS ept 3-11	2147.5 + 1078.4	3199.4
Dyn 1-16 + WS ept 1-10	2019.4 + 1207.2	3227.6
Dyn 1-17 + WS ept 1-10	2147.5 + 1207.2	3355.7
Dyn 1-17 + WS ept 1-11	2147.5 + 1278.3	3426.8
Dyn 5-17 + NMDA ept 16-29	1723.1 + 1564.6	3288.7
Dyn 2-11 + NMDA ept 14-32	1199.5 + 2128.0	3328.5
Dyn 5-17 + NMDA ept 8-21	1723.1 + 1672.8	3396.9
Dyn 5-17 + NMDA ept 16-31	1723.1 + 1722.7	3446.8
Dyn 5-17 + NMDA ept 12-26	1723.1 + 1751.8	3475.9
Dyn 1-17 + NMDA ept 12-26	1723.1 + 1751.8	3475.9
Dyn 1-17 + NMDA ept 14-38	2147.5 + 2892.2	5040.7
Dyn 1-17 + NMDA ept 1-25	2147.5 + 2892.2	5170.5

To determine whether KVNSEEEEEEDA can prevent the interaction of dynorphin peptides with the NR1 subunit of the NMDA receptor *in vitro*, we used voltage clamp recording methods to measure glutamate-evoked currents in *Xenopus* oocytes expressing NR1A/NR2B receptors. In the presence of glycine, application of glutamate (50 μ M) rapidly activated inward currents, an effect that was prevented by the NMDA receptor channel blocker MK-801. As previously reported for recombinant NMDA receptors, glutamate (50 μ M) elicited little current in the absence of added glycine. However, the addition of either 10 μ M dynorphin A or dynorphin (2-17) (Fig. 2A and Fig. 2B) to the bathing solution increased the amplitude of glutamate-evoked currents to $165 \pm 23\%$ of control values. The co-application of increasing concentrations of either D- or L-KVNSEEEEEEDA reduced the potentiation of glutamate currents evoked by the dynorphin peptides (Fig. 2C and 2D). KVNSEEEEEEDA failed to modify glutamate-evoked currents in the absence of dynorphin peptides. Similarly, it did not modify (³H)TCP binding to the NMDA receptor complex in cortical membranes indicating that the inhibitory effects of KVNSEEEEEEDA did not result from a direct interaction with NMDA receptors.

The neurotoxic actions of the dynorphins have been attributed to the activation of NMDA receptors and resulting excitotoxicity. To determine whether KVNSEEEEEEDA is neuroprotective *in vitro*, primary cortical cultures were exposed to dynorphin (2-17) in the presence or absence of KVNSEEEEEEDA. Exposure of cortical cultures to increasing
5 concentrations of dynorphin (2-17) caused significant cell death as indicated by increases in lactate dehydrogenase (LDH) activity and reduction in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) concentrations. Neither the D- nor L-forms of KVNSEEEEEEDA affected LDH or MTT levels. When, however, cultures were exposed to dynorphin (2-17) in the presence of D- or L- KVNSEEEEEEDA, the neurotoxic effects of
10 dynorphin A (2-17) were significantly reduced (Fig. 3A and 3B).

Excessive NMDA receptor activation has been implicated in neuronal injury caused by stroke. Although direct-acting NMDA receptor antagonists reduce injury in experimental models of cerebral ischemia, neurotoxicity precludes their clinical use. Since dynorphin A levels are increased during ischemic brain injury (R.T. Cheung & D.F.
15 Cechetto 1995 *J Comp Neurol* 362:535-550) and dynorphin A-related peptides enhance NMDA receptor-mediated responses, we next examined whether KVNSEEEEEEDA can protect against nerve injury in rats using an established ischemia model. Rats received an intracerebroventricular infusion of either L-KVNSEEEEEEDA (10-150 nmol), D-KVNSEEEEEEDA (150 nmol) or an equivalent volume of vehicle. Thirty min later, the
20 carotid arteries and right middle cerebral artery were occluded for 60 min as previously described (Chen, S.T. *et al.* 1987 *Neurology* 37:1227-1229). The rats were killed 48 hr after reperfusion and brain injury volume was evaluated by 2,3,5-triphenyletrazolium chloride (TTC) staining. Infarction of the cortex was seen in all control animals. However, the size and infarct volume were significantly reduced in animals that had received
25 KVNSEEEEEEDA prior to occlusion (Fig. 3C-E). KVNSEEEEEEDA pre-treatment also increased locomotor activity in stroke animals and reduced ischemia-induced body asymmetry as examined by the elevated body swing test (Fig. 3F).

Levels of dynorphin A and related peptides increase significantly following spinal cord trauma and this increase is thought to contribute to neuronal death and
30 neurodegeneration associated with secondary injury. To determine whether KVNSEEEEEEDA also prevent cytotoxic actions of dynorphin peptides in the spinal cord, we isolated neurons from the spinal cord of embryonic neurons and used time lapse photography to examine neuronal viability following exposure to dynorphin (2-17) and

KVNSEEEEEEDA. Neuronal viability was assessed by repeatedly photographing the same neurons at 24 hrs using a Nikon Diaphot inverted microscope with phase contrast optics and neurons counted as previously described (Hauser, K.F. *et al.* 1999 *Exp Neurol* **160**:361-375). At 24-72 hrs in culture, large numbers of small multipolar cells that displayed a neuronal phenotype and NMDAR1 immunofluorescence were seen (Hauser, K.F. *et al.* 1999 *Exp Neurol* **160**:361-375). Continuous exposure of cultures to 100 μ M dynorphin A(2-17) causes profound neuronal loss over time, an effect that is attenuated by NMDA receptor antagonists, but not opioid receptor antagonists (Hauser, K.F. *et al.* 1999 *Exp Neurol* **160**:361-375). A significant increase in cell survival was observed in cultures exposed to equimolar concentrations of dynorphin (2-17) and D-KVNSEEEEEEDA (Fig. 4A).

The intrathecal injection of dynorphin A or dynorphin A(2-17) induces long-lasting allodynia, a condition in which normally non-noxious stimuli elicit pain. Higher doses or sustained exposure to these peptides induce hind-limb paralysis and a loss of neuronal cell bodies in the lumbosacral spinal cord. To determine whether KVNSEEEEEEDA can protect spinal cord neurons *in vivo*, we examined whether the intrathecal infusion of KVNSEEEEEEDA prevents the tactile allodynia elicited by dynorphin (2-17). Rats were implanted with an intrathecal cannula as previously described (Yaksh, T.L. & Rudy, T.A. 1976 *Physiol Behav* **17**:1031-1036; Ren, K. 1999 *Physiol Behav* **67**:711-716). Tactile allodynia was assessed 1, 3 and 7 days following the intrathecal infusion of dynorphin A(2-17) (5 nmol), KVNSEEEEEEDA (30 – 250 nmol), or their combination using Von Frey filaments.

A significant increase in nociceptive threshold was apparent in all rats that received dynorphin A(2-17). In rats that received dynorphin A(2-17) and KVNSEEEEEEDA (250 nmol), nociceptive thresholds did not differ from that of vehicle-treated rats (Fig. 4B). To evaluate whether KVNSEEEEEEDA can also prevent the motor dysfunction produced by a higher dose of dynorphin (2-17), rats received an intrathecal infusion of dynorphin (2-17) (75 nmol), KVNSEEEEEEDA (30-250 mol) or their combination. The presence or absence of hind-limb paralysis was assessed over a 72 hr period and cumulative paralysis scores were calculated for each treatment group. All dynorphin (2-17)-treated rats exhibited hind-limb paralysis within 5 min of infusion. When, however, dynorphin A(2-17) was administered in combination with either D- or L- KVNSEEEEEEDA, a dose-related reduction in the incidence and duration of flaccid paralysis was seen (Fig. 4C and Fig. 5A).

The ability of KVNSEEEEEEDA to protect against motor impairment was also assessed in studies examining the RotaRod performance of rats for three weeks after the intrathecal administration of dynorphin A(2-17) and KVNSEEEEEEDA (250 nmol). Although infusion of dynorphin A(2-17) alone significantly impaired RotaRod performance, no such impairment occurred in animals that received dynorphin A(2-17) with KVNSEEEEEEDA (Fig. 4D).

A significant increase in nociceptive threshold was apparent in all rats that received dynorphin A(2-17). In rats that received dynorphin A(2-17) and KVNSEEEEEEDA (250 nmol), nociceptive thresholds did not differ from that of vehicle-treated rats (Fig. 4B). To evaluate whether KVNSEEEEEEDA can also prevent the motor dysfunction produced by a higher dose of dynorphin (2-17), rats received an intrathecal infusion of dynorphin (2-17) (75 nmol), KVNSEEEEEEDA (30-250 mol) or their combination. The presence or absence of hind-limb paralysis was assessed over a 72 hr period and cumulative paralysis scores were calculated for each treatment group. All dynorphin (2-17)-treated rats exhibited hind-limb paralysis within 5 min of infusion. When, however, dynorphin A(2-17) was administered in combination with either D- or L- KVNSEEEEEEDA, a dose-related reduction in the incidence and duration of flaccid paralysis was seen (Fig. 4C and Fig. 5A). The ability of KVNSEEEEEEDA to protect against motor impairment was also assessed in studies examining the RotaRod performance of rats for three weeks after the intrathecal administration of dynorphin A(2-17) and KVNSEEEEEEDA (250 nmol). Although infusion of dynorphin A(2-17) alone significantly impaired RotaRod performance, no such impairment occurred in animals that received dynorphin A(2-17) with KVNSEEEEEEDA (Fig. 4D). When KVNSEEEEEEDA was given 2 to 5 minutes after dynorphin induced paralysis, it reversed dynorphin-evoked tactile allodynia and dynorphin-induced motor paralysis (Fig. 5B).

The motor dysfunction produced by high doses of dynorphin A(2-17) is associated with a loss of neurons in the spinal cord. We performed immunocytochemical studies to identify specific neuronal subpopulations that are targeted by dynorphin A(2-17) and determine whether neuronal death is prevented by intrathecal infusion of KVNSEEEEEEDA. Intrathecal infusion of dynorphin A(2-17) resulted in a selective loss of neurons expressing PKC γ in the dorsal horn as well as motor neuron loss. These effects were apparent for at least 3 days following infusion. In rats that had received KVNSEEEEEEDA in combination with dynorphin A(2-17), sparing of PKC γ and motor neurons was seen.

Chronic administration of opiates results in tolerance to their analgesic effects. Tolerance has been attributed to increased activity of spinal dynorphin systems and the NMDA receptor dependent mechanisms. Consistent with this hypothesis, administration of dynorphin antiserum or NMDA receptor antagonists attenuates antinociceptive tolerance to morphine. Figure 6 shows that chronic exposure to morphine (s.c. implantation of 75 mg morphine pellet) results in tolerance to the analgesic effects of intrathecal morphine. In animals, however, that received an intrathecal infusion of KVNSEEEEEEDA 10 min prior to intrathecal morphine, the expression of antinociceptive tolerance to morphine was prevented.

EXAMPLE 1

Peptides. The following epitopes of NR1 were synthesized, KVNSEEEEEEDA (res. 590-600 of SEQ ID NO: 2), and a larger (51-mer) epitope MLYLLDRFSP FGRFKVNSEEEEDALTLSS AMWFSWGVLL NSGIGEGAPRS (res. 576-626 of SEQ ID NO: 2). The above peptides were made by the Sequencing and Synthesis Laboratory at the Johns Hopkins School of Medicine, Baltimore MD. Dynorphins (1-17) and (2-17) were purchased from Sigma (Saint Louis, MO).

Digest. A digest of the equimolar mixture of the KVNSEEEEEEDA + the 51-mer + dynorphin (1-17) was done as previously described (Woods, A.S. & Huestis, M.A. 2001 *JASMS* 12:88-96; Pole Bio-Informatique Lyonnais: http://pbil.ibcp.fr/cgi-bin/secpred_sopma.pl). Chymotrypsin (0.5 µg/µl) from Roche Diagnostics (Indianapolis, IN) was used, an overnight time point was added.

Mass Spectrometry. Spectra were acquired on a MALDI DE-Pro from Applied-Biosystem, equipped with a nitrogen laser (337 nm) as previously described (Woods, A.S. & Huestis, M.A. 2001 *JASMS* 12:88-96). Aliquots were also analyzed using a Q-TOF (Micromass, Manchester England) as previously described (Sobott, F. *et al.* 2002 *J Biol Chem* 277:38921-38929).

Animals and Drug Administration. Adult Sprague-Dawley rats (body weight = 336 ± 7 g), purchased from the Charles River Laboratories, were anesthetized with chloral hydrate (0.4 g/kg, i.p.). Animals were placed in a stereotaxic frame. L-KVNSEEEEEEDA (10 nmol in 10 µl, 30 nmol in 30 µl, 150 nmol in 30 µl), D-KVNSEEEEEEDA (150 nmol in 30 µl) or vehicle (0.9% saline or water, 30 µl) were administered through a Hamilton syringe over 10 minutes intracerebrocortically. The coordinates were: 0.8 mm posterior to the bregma, 1.5 mm lateral to the midline, and -3.5 mm below dura surface. The speed of

injection was controlled by a syringe pump at a speed of 2.5 μ l/min. The needle was retained in place for 5 min after injection. A piece of bone wax (W810, Ethicon) was applied to the skull defect to prevent the leakage of the solution after injection.

Brain Ischemia/Reperfusion. Thirty minutes after intracerebral administration of KVNSEEEEEEDA or vehicle, the anesthetized animals were subjected to cerebral ischemia. The ligation of the right middle cerebral artery (MCA) and bilateral common carotids (CCAs) was performed using methods previously described (Chen, S.T. *et al.* 1987 *Neurology* 37:1227-1229). The CCAs were ligated with non-traumatic arterial clips. The right MCA was ligated with 10-O suture. After 60 minutes of ischemia, the suture on the MCA and arterial clips on CCAs were removed to allow reperfusion. Core body temperature was monitored with a thermistor probe and maintained at 37°C with a heating pad during anesthesia. After recovery from the anesthesia, body temperature was further maintained at 37°C using a heat lamp.

Locomotor Behavioral Measurements. Animals were placed in an Accuscan activity monitor (Columbus, OH) 2 days after ischemia for behavioral recording. The monitor contained 16 horizontal and 8 vertical infrared sensors spaced 2.5 cm apart. The vertical sensors were situated 10.5 cm from the floor of the chamber. Each animal was placed in a 42 x 42 x 31 cm Plexiglas open box for one hour. Motor activities, such as distance traveled, vertical and horizontal movement were calculated by the number of beams broken by the animals from 0 to 30 min after placement in the chamber.

Triphenyltetrazolium Chloride (TTC) Staining. Two days after MCA ligation, some animals were sacrificed and perfused intracardially with saline. The brain tissue was then removed, immersed in cold saline for 5 minutes, and sliced into 2.0-mm thick sections. The brain slices were incubated in a 2% triphenyltetrazolium chloride (TTC, Sigma) dissolved in normal saline for 15 minutes at room temperature, and then transferred into a 5% formaldehyde solution for fixation. The area of infarction on each brain slice was measured double blind using a digital scanner and the Image Tools program (University of Texas Health Sciences Center, San Antonio). The total infarction volume in each animal was obtained from the product of average slice thickness (2 mm) and sum of the area of infarction in all brain slices.

Post-Stroke data. d-KVNSEEEEEEDA was given within 30 min after middle cerebral artery ligation in 23 rats. Of these, 11 rats received 150 nmol (150 nmole/25 μ l x 25 μ l) and the other 12 rats received 416 nmol (500 nmol/30 μ L x 25 μ L) of d-

KVNSEEEEEEDA. We found that d-KVNSEEEEEEDA, given after stroke, did not significantly reduce the volume of infarction ($p=0.577$, $F(2,48)=0.556$, One way ANOVA). However, d-KVNSEEEEEEDA increased locomotor movement in stroke animals. There is a significant correlation between the log dose of d-KVNSEEEEEEDA given and total distance traveled 2 days after stroke ($P=0.039$, $R = 0.323$, total distance traveled = $100.233 + (13.796 * \log [KVNSEEEEEEDA])$). There is also a trend showing that movement time was enhanced in the stroke animals that received post-stroke d-KVNSEEEEEEDA ($P=0.062$, $R = 0.294$, Movement time = $100.305 + (10.697 * \log [d-KVNSEEEEEEDA])$). These data indicate that d-KVNSEEEEEEDA, given early after ischemic insults, is envisioned as improving locomotor activity in stroke animals.

While the present invention has been described in some detail for purposes of clarity and understanding, one skilled in the art will appreciate that various changes in form and detail can be made without departing from the true scope of the invention. All figures, tables, and appendices, as well as patents, applications, and publications, referred to above, are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1. A molecular decoyant for treatment of an organism which has been subjected to a dynorphin which exerts a neurotoxic effect only after first binding to an endogenous NMDA receptor and which exerts an analgesic effect only after first binding to
5 an endogenous opioid receptor, said treatment being for the reduction of said neurotoxic effect without preventing said analgesic effect, having a chemical structure which functionally resembles the ligand binding site of said endogenous NMDA receptor for the dynorphin, and which does not functionally resemble the ligand binding site of said endogenous opioid receptor for the dynorphin, said decoyant being a substance selected
10 from the group consisting of:

(a) a substance which is a portion of the NR1 subunit of said endogenous NMDA receptor for said dynorphin, said portion being (i) sufficiently small so as not to elicit an autoimmune response against said endogenous NMDA receptor when administered *in vivo*, and (ii) not substantially larger than the smallest size
15 needed to retain the elements of the binding site of said endogenous NMDA receptor which are essential for retaining the ability of said endogenous NMDA receptor to selectively and specifically recognize and bind to said dynorphin with an affinity permitting the competition with binding of said dynorphin to said endogenous NMDA receptor and not having the elements of the binding site of said
20 endogenous opioid receptor which are essential for retaining the ability of said endogenous opioid receptor to selectively and specifically recognize and bind to said dynorphin with an affinity so as not to permit a competition with binding of said dynorphin to said endogenous opioid receptor;

(b) a substance having a chemical structure which substantially corresponds to that of said substance of (a), synthesized by chemical and/or recombinant DNA techniques and having the ability to selectively and specifically bind to the dynorphin without eliciting an autoimmune response against said endogenous NMDA receptor when administered *in vivo*; and

(c) a substance having a chemical structure consisting essentially of the chemical structure of the substance of (a) or (b).
30

2. The molecular decoyant of claim 1, whereby said portion of the NR1 subunit of said endogenous NMDA receptor is residue 594-599.

3. The molecular decoyant of claim 1, wherein said molecular decoyant is a substance of (a).

4. The molecular decoyant of claim 3, wherein said molecular decoyant is a peptide chain expressed by cells genetically engineered by recombinant DNA techniques.

5 5. The molecular decoyant of claim 3, wherein said molecular decoyant is a synthetic peptide or peptidomimetic.

6. The molecular decoyant of claim 3, wherein said molecular decoyant includes the amino acid sequence EEEEEED or KVNSEEEEEEDA.

7. The molecular decoyant of claim 3, wherein said organism is an animal.

10 8. The molecular decoyant of claim above, wherein said animal is a human.

9. The molecular decoyant of claim 1, wherein said molecular decoyant is a substance of (b).

10. The molecular decoyant of claim 9, wherein said molecular decoyant is a peptide chain expressed by cells genetically engineered by recombinant DNA techniques.

15 11. The molecular decoyant of claim 9, wherein said molecular decoyant is a synthetic peptide or peptidomimetic.

12. The molecular decoyant of claim 9, wherein said molecular decoyant includes an amino acid sequence substantially corresponding to EEEEEED or KVNSEEEEEEDA.

20 13. The molecular decoyant of claim 9, wherein said organism is an animal.

14. The molecular decoyant of claim above, wherein said animal is a human.

15. A method for the treatment of an organism which has been subjected to a dynorphin which exerts a neurotoxic effect only after first binding to an endogenous NMDA receptor and which exerts an analgesic effect only after first binding to an endogenous opioid receptor, said treatment being for the reduction of said neurotoxic effect without preventing said analgesic effect, comprising:

30 (a) administering to said organism, in a quantity sufficient to reduce said neurotoxic effect without preventing said analgesic effect, a molecular decoyant having a chemical structure which functionally resembles the ligand binding site of said endogenous NMDA receptor for the dynorphin, and which does not functionally resemble the ligand binding site of said endogenous opioid receptor for the dynorphin, said decoyant being a substance selected from the group consisting of:

(b) a substance which is a portion of the NR1 subunit of said endogenous NMDA receptor for said dynorphin, said portion being (i) sufficiently small so as not to elicit an autoimmune response against said endogenous NMDA receptor when administered *in vivo*, and (ii) not substantially larger than the smallest size
5 needed to retain the elements of the binding site of said endogenous NMDA receptor which are essential for retaining the ability of said endogenous NMDA receptor to selectively and specifically recognize and bind to said dynorphin with an affinity permitting the competition with binding of said dynorphin to said endogenous NMDA receptor and not having the elements of the binding site of said
10 endogenous opioid receptor which are essential for retaining the ability of said endogenous opioid receptor to selectively and specifically recognize and bind to said dynorphin with an affinity so as not to permit a competition with binding of said dynorphin to said endogenous opioid receptor;

(c) a substance having a chemical structure which substantially corresponds
15 to that of said substance of (a), synthesized by chemical and/or recombinant DNA techniques and having the ability to selectively and specifically bind to the dynorphin without eliciting an autoimmune response against said endogenous NMDA receptor when administered *in vivo*; and

(d) a substance having a chemical structure consisting essentially of the
20 chemical structure of the substance of (a) or (b).

16. Use of a molecular decoyant for the manufacture of a medicament for the treatment of an organism which has been subjected to a dynorphin which exerts a neurotoxic effect only after first binding to an endogenous NMDA receptor and which exerts an analgesic effect only after first binding to an endogenous opioid receptor, said
25 treatment being for the reduction of said neurotoxic effect without preventing said analgesic effect, having a chemical structure which functionally resembles the ligand binding site of said endogenous NMDA receptor for the dynorphin, and which does not functionally resemble the ligand binding site of said endogenous opioid receptor for the dynorphin, said decoyant being a substance selected from the group consisting of:

30 (a) a substance which is a portion of the NR1 subunit of said endogenous NMDA receptor for said dynorphin, said portion being (i) sufficiently small so as not to elicit an autoimmune response against said endogenous NMDA receptor when administered *in vivo*, and (ii) not substantially larger than the smallest size

needed to retain the elements of the binding site of said endogenous NMDA receptor which are essential for retaining the ability of said endogenous NMDA receptor to selectively and specifically recognize and bind to said dynorphin with an affinity permitting the competition with binding of said dynorphin to said endogenous NMDA receptor and not having the elements of the binding site of said endogenous opioid receptor which are essential for retaining the ability of said endogenous opioid receptor to selectively and specifically recognize and bind to said dynorphin with an affinity so as not to permit a competition with binding of said dynorphin to said endogenous opioid receptor;

(b) a substance having a chemical structure which substantially corresponds to that of said substance of (a), synthesized by chemical and/or recombinant DNA techniques and having the ability to selectively and specifically bind to the dynorphin without eliciting an autoimmune response against said endogenous NMDA receptor when administered *in vivo*; and

(c) a substance having a chemical structure consisting essentially of the chemical structure of the substance of (a) or (b).

17. The method of claim 15, wherein said method further comprises administering said molecular decoyant together with dynorphin to reduce the neurotoxic effect of said administered dynorphin.

18. The use of claim 16, wherein said use further comprises use of said molecular decoyant together with dynorphin to reduce the neurotoxic effect of said administered dynorphin.

19. The method of claim 15, wherein said method further comprises administering said molecular decoyant in the absence of dynorphin to reduce the neurotoxic effect of non-administered dynorphin.

20. The use of claim 16, wherein said use further comprises use of said molecular decoyant in the absence of dynorphin to reduce the neurotoxic effect of non-administered dynorphin.

21. The method of claim 15, wherein said method further comprises administering said molecular decoyant to a patient with stroke or spinal cord injury to reduce the neurotoxic effect of dynorphin.

22. The use of claim 16, wherein said use further comprises use of said molecular decoyant for administration to a patient with stroke or spinal cord injury to reduce the neurotoxic effect of dynorphin.

23. The method of claim 15, wherein said method further comprises
5 administering said molecular decoyant to a patient in a neuropathic pain state to reduce the neurotoxic effect of dynorphin.

24. The use of claim 16, wherein said use further comprises use of said molecular decoyant for administration to a patient in a neuropathic pain state to reduce the neurotoxic effect of dynorphin.

10 25. The method of claim 15, wherein said method further comprises administering said molecular decoyant to a patient at risk for antinociceptive tolerance to morphine to reduce said risk.

26. The use of claim 16, wherein said use further comprises use of said molecular decoyant for administration to a patient at risk for antinociceptive tolerance to
15 morphine to reduce said risk.

27. The method of claim 15 or use of claim 16, whereby said portion of the NR1 subunit of said endogenous NMDA receptor is residue 594-599.

28. The method of claim 15 or use of claim 16, wherein said molecular decoyant is a substance of (a).

20 29. The method or use of claim 28, wherein said molecular decoyant is a peptide chain expressed by cells genetically engineered by recombinant DNA techniques.

30. The method of use of claim 28, wherein said molecular decoyant is a synthetic peptide or peptidomimetic.

25 31. The method or use of claim 28, wherein said molecular decoyant includes the amino acid sequence EEEEEED or KVNSEEEEEEDA.

32. The method of use of claim 28, wherein said organism is an animal.

33. The method or use of claim above, wherein said animal is a human.

34. The method of claim 15 or use of claim 16, wherein said molecular decoyant is a substance of (b).

30 35. The method of use of claim 34, wherein said molecular decoyant is a peptide chain expressed by cells genetically engineered by recombinant DNA techniques.

36. The method or use of claim 34, wherein said molecular decoyant is a synthetic peptide or peptidomimetic.

37. The method or use of claim 34, wherein said molecular decoyant includes an amino acid sequence substantially corresponding to EEEEEED or KVNSEEEEEEDA.
38. The method or use of claim 34, wherein said organism is an animal.
39. The method or use of claim above, wherein said animal is a human.
- 5 40. A pharmaceutical composition comprising a molecular decoyant in accordance with any of claims 1-14 in an amount effective to reduce the neurotoxic effect of dynorphin without preventing its analgesic effect, and a pharmaceutically acceptable excipient.

Fig. 1

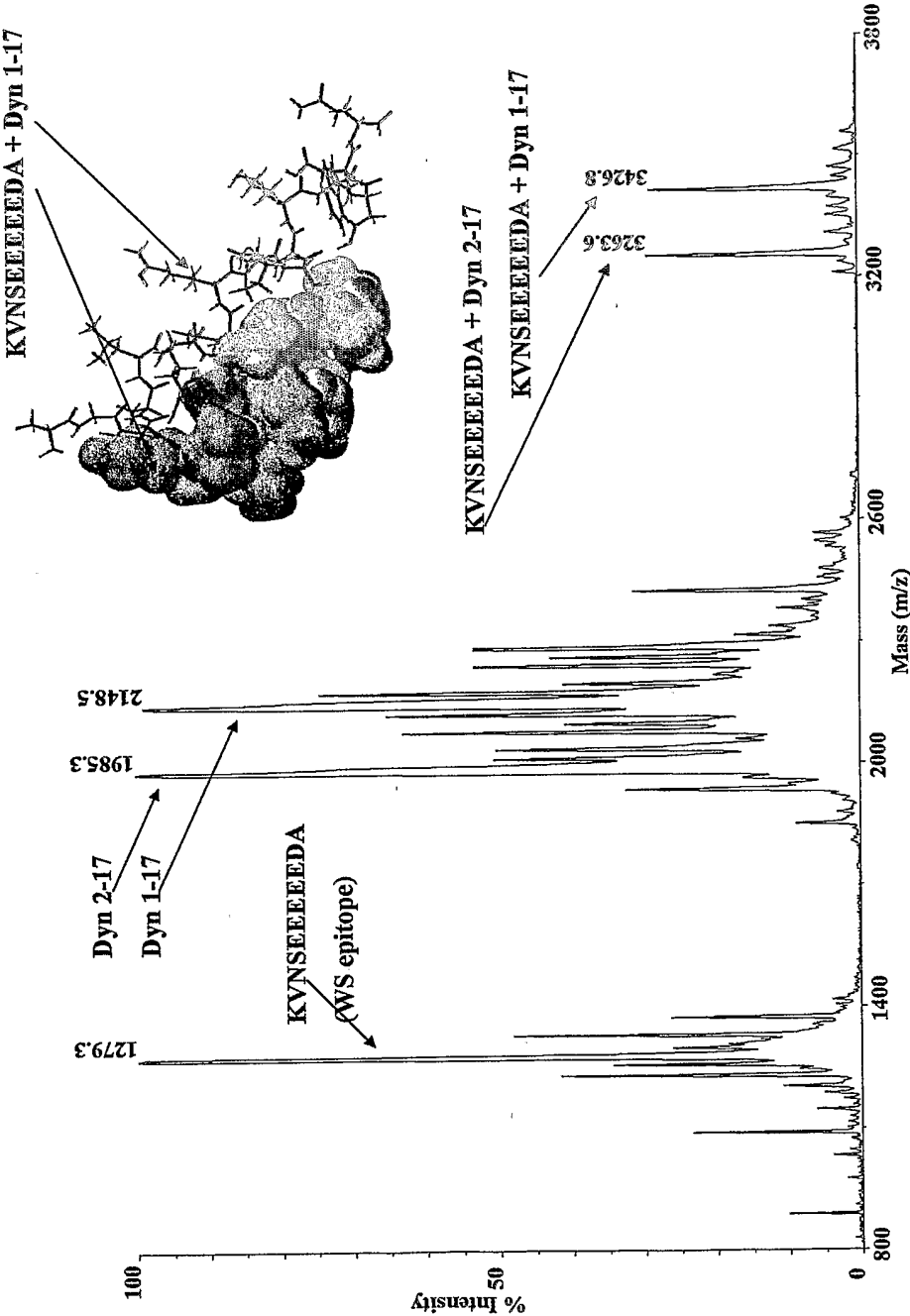


Fig. 2

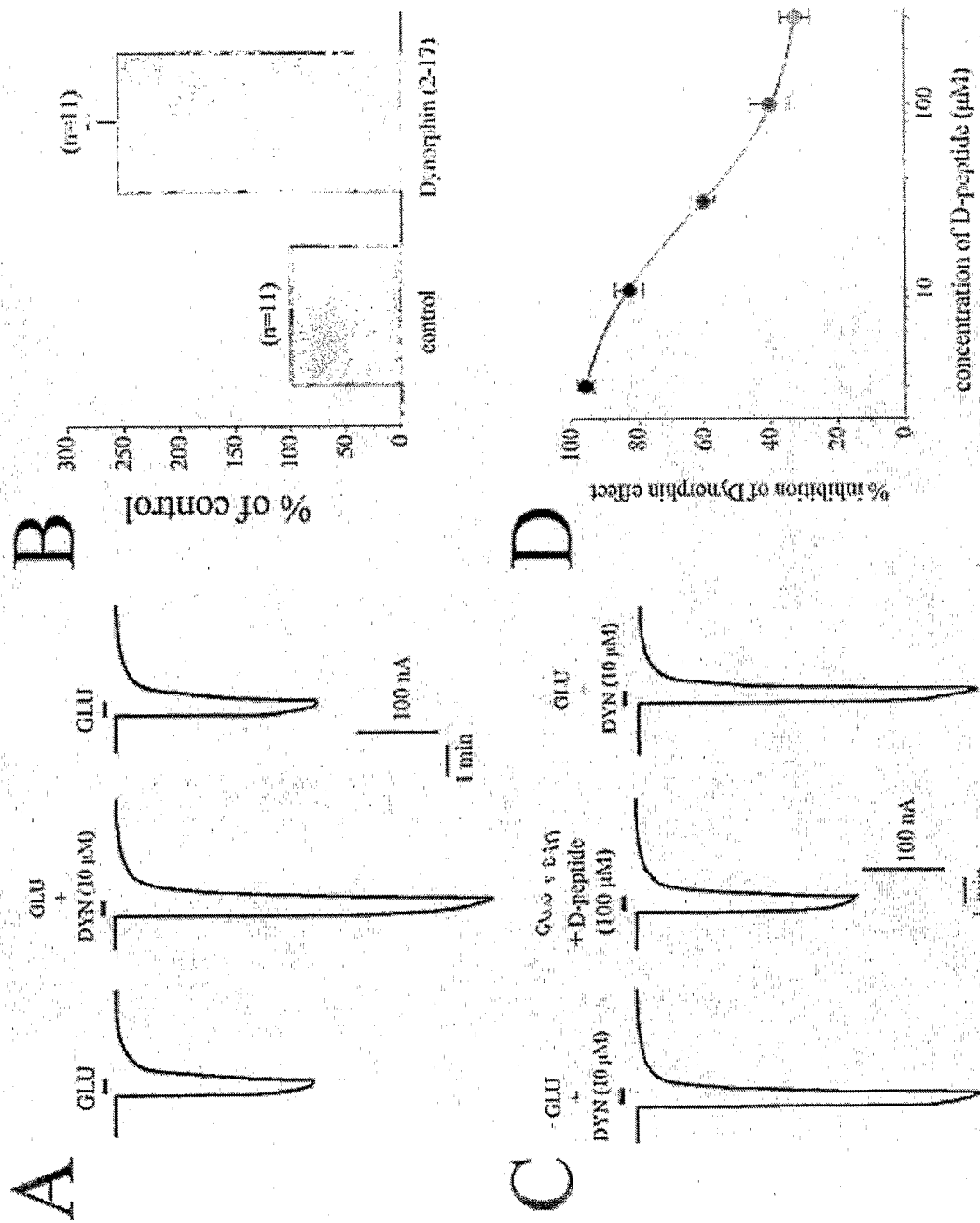


Figure 3

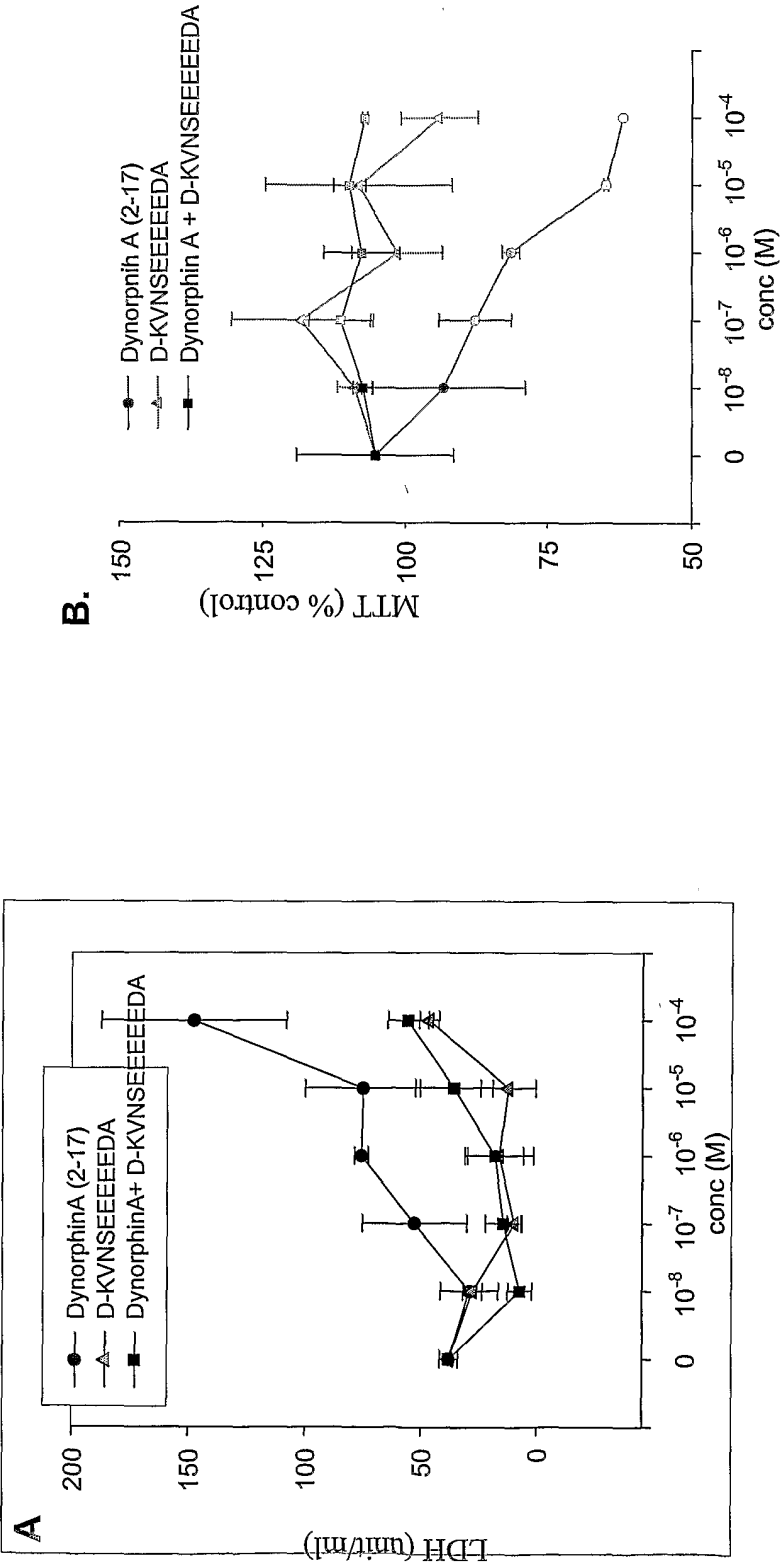


Fig. 3(C-F)

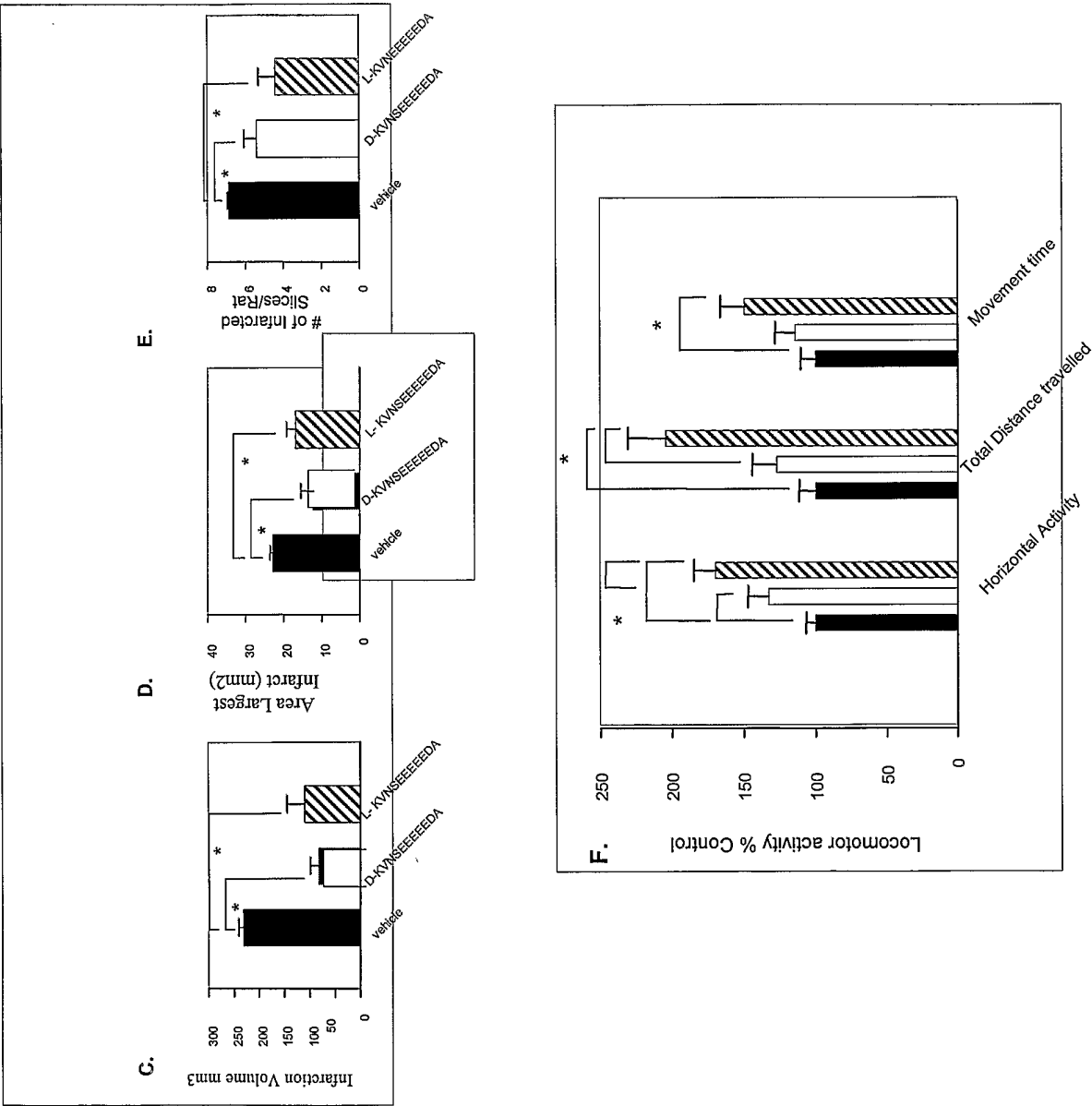


Figure 4

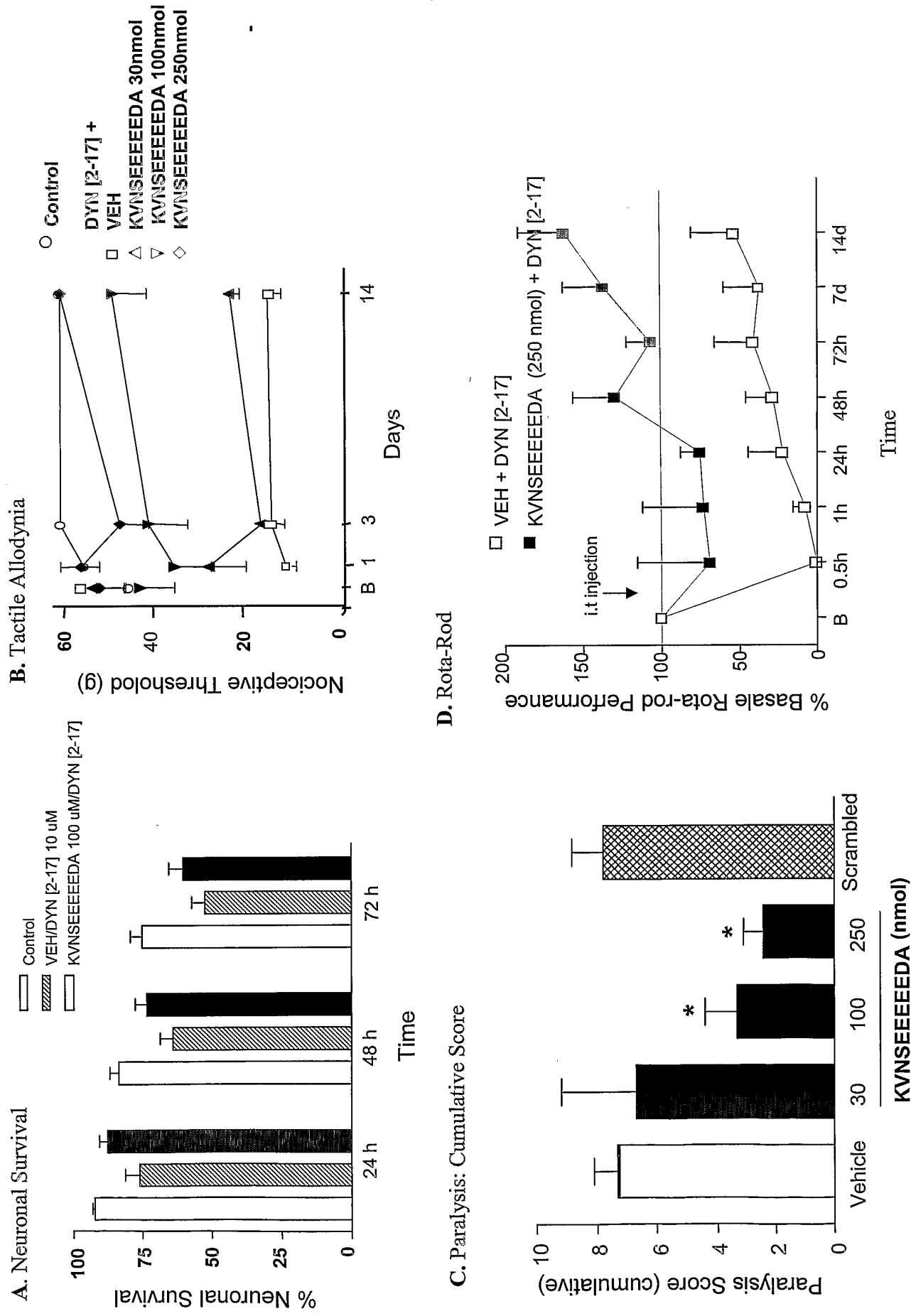


Fig. 5A

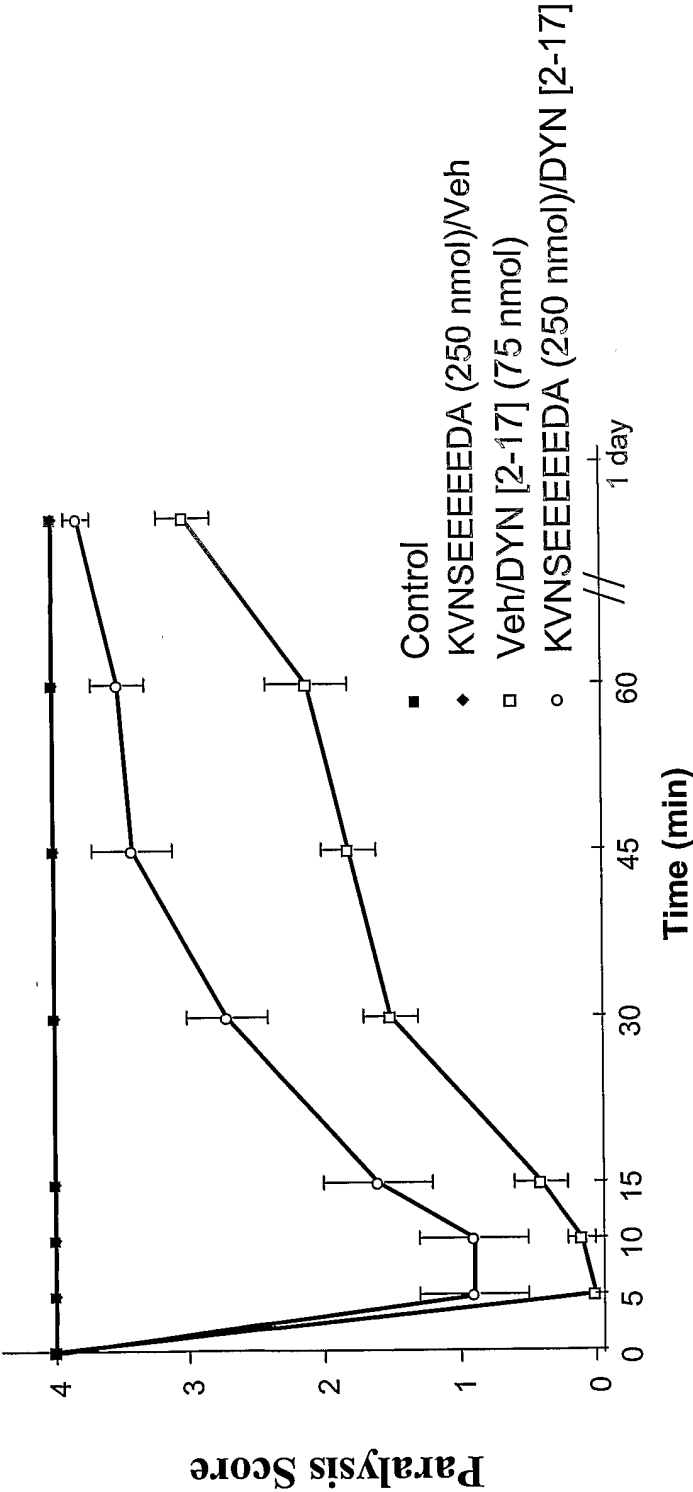


Fig. 5B

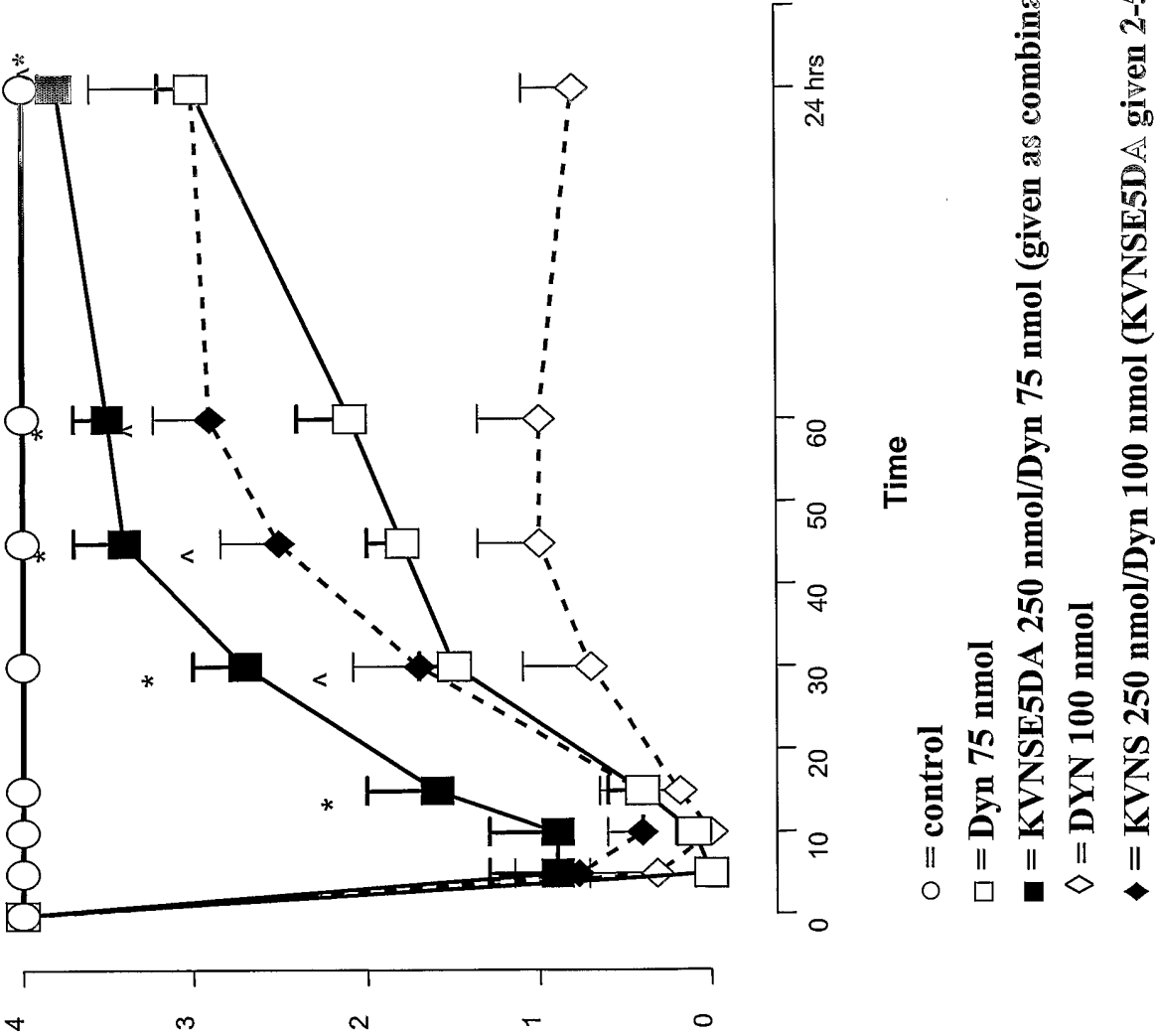
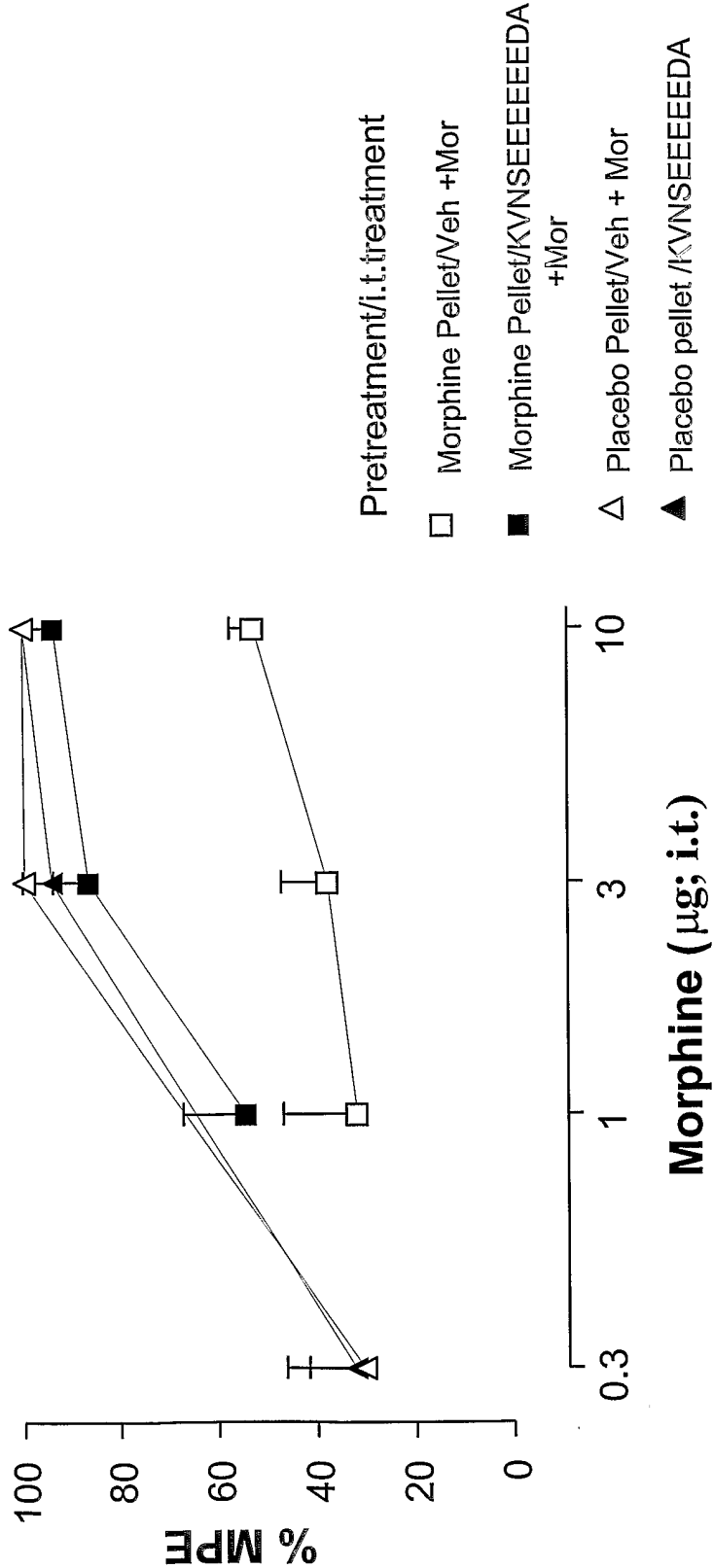


Fig. 6



SEQUENCE LISTING

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Health and Human Services
Woods, Amina S.
Shippenberg, Toni S.
Sharpe, Lawrence

<120> IDENTIFICATION AND TARGETING OF
CONSENSUS SITES BY WHICH DYNORPHIN EXERTS ITS NEUROTOXIC
EFFECTS ENABLES ANALGESIA WITHOUT NEUROTOXICITY

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