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(71) Applicant *(for all designated States except US)*: **UNIVERSITY OF FLORIDA RESEARCH FOUNDATION, INC.** [US/US]; 223 Grinter Hall, Gainesville, Florida 32611.

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **CAUDLE, Robert, M.** [US/US]; 5121 Sw 82nd Terrace, Gainesville, Florida 32608 (US).

(74) Agents: **VAN DYKE, Timothy, H.** et al.; Beusse Wolter Sanks Mora & Maire, 390 N. Orange Avenue, Ste. 2500, Orlando, FL 32801 (US).

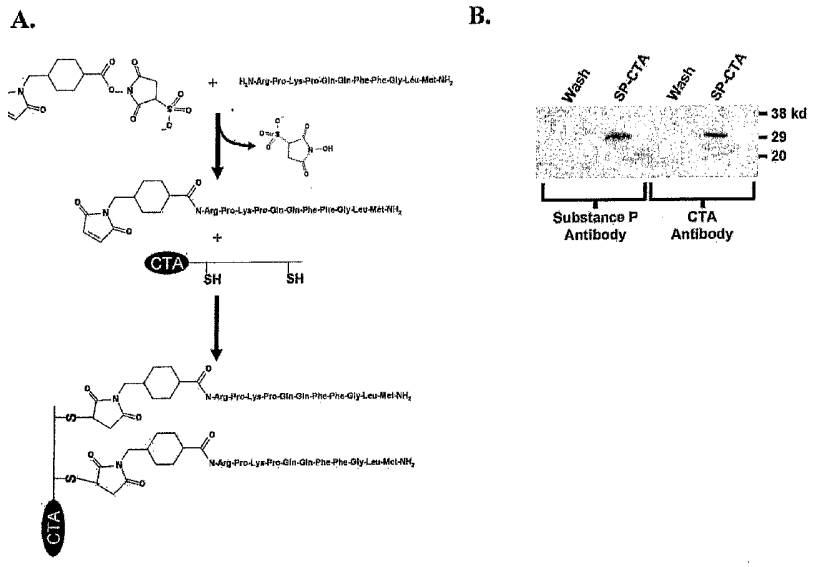
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(54) Title: TARGETED CHOLERA TOXIN FOR TREATMENT OF PERSISTENT OR CHONIC PAIN



(57) Abstract: Disclosed herein is a compound engineered by conjugating a cholera toxin (or subunit thereof), to a targeting molecule that assists in targeting the cholera toxin to a specific g-protein coupled receptor on neurons. Targeting molecules disclosed herein may include but are not limited to, substance P, an opioid, and CGRP, or any peptide for which there is a g-protein coupled receptor. Specifically exemplified is the engineering of a compound involving conjugating Substance P to a cholera toxin, wherein the compound suppresses pain in a subject while minimizing cell death of neurons.

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TARGETED CHOLERA TOXIN FOR TREATMENT OF PERSISTENT OR CHRONIC PAIN

Related Applications

[01] This application claims the benefit of U. S. Serial No. 60/669,802 filed April 9, 2005, under 35 USC § 119(e) which is incorporated herein by reference.

Background

[02] According to the National Institute of Neurological Disorders and Stroke typical pain, known as acute pain, is a normal sensation triggered in the nervous system to alert you of possible injury and the need to take care of yourself. Chronic pain differs from acute pain in that it persists. The American Chronic Pain Association (ACPA) estimates that one in three Americans (approximately 50 million people) suffered from some type of chronic pain in the past year. Chronic pain is treatable, but still poses a challenge. Pain management and treatments range from analgesics to using relaxation and imagery as a tool for distraction[4]. Some methods are more effective than others. Many of the medications used for pain management have negative side effects.

[03] Opioids are used for treatment of chronic pain, especially morphine which is commonly found in hospital settings. Although this narcotic induces sedation as well as pain relief it has many negative side effects. In addition to it being very addicting, morphine has frequently been associated with side effects such as nausea, vomiting, and constipation[4]. Studies conducted have also demonstrated the immunosuppressive effect of morphine, weakening the immune system of patients consuming the substance[8]. Long term use of the narcotic can severely damage a person's immune system increasing their risk of infection and other ailments. Other experiments have found that in smaller doses morphine's negative side effects can be minimized, but are none-the-less evident. Limiting the dosage unfortunately resulted in less pain blockage[4]. Morphine has helped those suffering to successfully cope with chronic pain, but due to its array of negative side effects and limitations other methods must be investigated.

[04]

[05] There have been attempts to develop a targeted pain suppressant. One attempt has involved the engineering of a substance P/Saporin conjugate. Though this conjugate appeared to decrease hyperalgesia induced by capsaicin, inflammation, and nerve injury[2], its utilization as a pain treatment is doubtful because such toxin conjugates kill the cells involved in hyperalgesia thereby resulting in negative long-term effects[3]. See U.S. Patent Publication 2004/0253248.

Brief Description of the Drawings

[06] **Figure 1.** Synthesis of SP-CTA. **A.** Schematic representation of the procedure used to synthesize SP-CTA. **B.** Western blots of final SP-CTA product. SP-CTA and the filtrate from the Centricon Plus-20 concentrating tubes (Wash) were run on western blots and probed with antibodies to substance P and the catalytic subunit of cholera toxin (CTA). The SP-CTA product reacted with both antibodies.

[07] **Figure 2.** In situ evaluation of SP-CTA. **A.** CHO cells stably transfected with the NK1 receptor were treated with either SP-CTA (1ìg/ml), CTA (1ìg/ml) or were not exposed to any agents (Control). The cells were cultured over night in these solutions and then immunocytochemistry was performed on the cells using an antibody to CTA and Rhodamine-tyramide amplification. **B.** Concentration response relationship for SP-CTA on cAMP production. CHO-NK1 cells were exposed to the indicated concentrations of SP-CTA for 1 hour. The cells were cultured for another 48 hours following washout of the SP-CTA with fresh culture media. The cells were harvested and cAMP content was measured using a commercial cAMP assay. (N = 5 100mm plates/ concentration) **C.** Time course of SP-CTA's effects on cAMP content of CHO-NK1 cells. The cells were incubated for 1 hour with 100 ng/ml SP-CTA and cultured in fresh media for the indicated times. The cells were then harvested and analyzed for cAMP content. (N = 5 100 mm plates/ treatment) **D.** Comparison of the effects of substance P (SP), the catalytic subunit of cholera toxin (CTA) and SP-CTA on cAMP production in CHO-NK1 cells. Cells were incubated with 100 ng/ml of CTA, SP, SP-CTA or received no treatment for 4 hours. The cells were then cultured for 48 hours in fresh media, harvested and assayed for cAMP content. (N = 5 100 mm plates/ treatment) **E.** Further comparison of the effects of SP, CTA and SP-CTA on cAMP production. Cells were treated as described in D except the agents remained in the culture media for the full 48 hours. The cells were then harvested and analyzed for cAMP content. (N = 5 100 mm plates/ treatment). Asterisks indicate $p < 0.05$ one way ANOVA followed by Dunnett's test.

[08] **Figure 3.** Effect of intrathecal administration of SP-CTA on Cyclic AMP Response Element Binding protein phosphorylation (pCREB). Rats (N = 6 per treatment group) received intrathecal injections of CTA (50 ìg), SP-CTA (50ìg) or Saline (20ìl). The animals were allowed to recover for 24 hours. The spinal cords were removed and prepared for immunocytochemistry using an antibody to pCREB, a secondary antibody coupled to HRP and diaminobenzidine. Phosphorylated CREB is visible as dark staining nuclei.

[09] **Figure 4.** Effect of intrathecal SP-CTA on thermal nociception in rats. **A.** SP-CTA dose response relationship. Rats (N = 10/ dose) received intrathecal injections of the indicated doses

of SP-CTA in 20 ìl of saline. Twenty four hours following the injections the animals were tested for thermal nociception using a Hargreaves apparatus 24. Asterisks indicate $p < 0.05$ one way ANOVA followed by Dunnett's test. **B.** Time course of intrathecal SP-CTA on thermal nociception. Rats (N = 20) were injected intrathecally with 1 ìg of SP-CTA in 20 ìl of saline. The animals were tested for thermal nociception at the times indicated. Zero represents the day of injection. Asterisk indicates $p < 0.05$ repeated measures ANOVA followed by Dunnett's test. Note that less restrictive paired ttests (1 tailed) indicate that days 1 ($p = 0.003$), 2 ($p = 0.01$), 3 ($p = 0.05$) and 4 (0.03) are significantly different from day 0.

Detailed Description

[010] In one embodiment, the invention relates to engineering of a compound involving conjugating a cholera toxin subunit A to a targeting molecule (TM) that assists in targeting the cholera toxin to a specific g-protein coupled receptor on neurons, or other cell types. Targeting molecules may include but are not limited to, substance P, an opioid, and CGRP, or any peptide for which there is a g-protein coupled receptor. Cholera toxin (CT) is a bacterial toxin secreted by Vibrio cholerae and comprising A and B subunits. The A subunit is the catalytic molecule and contributes to intracellular toxicity and the B subunit is required for binding of CT to a cell surface receptor. The structural genes encoding A and B subunits are designated as ctxA and ctxB respectively. (see, e.g., Kaper and Srivastava, Indian J. Med. Res.95:163-7 (1992); Field, Am. J. Clin. Nutr. (1):189-96 (1979); and Van Heyningen et al., Ciba Found Symp. 1976;(42):73-88 (1976)

[011] Cholera toxin subunit A (CTA) is responsible for the toxic effect induced by this toxin. It is an ADP-ribosyltransferase that disrupts the proper signaling of G protein activity. The non-toxic B subunit of cholera toxin, choleragenoid, is responsible for the uptake and transport of the toxin into the axon and cell bodies[1]. The A subunit of cholera toxin is preferably, though not necessarily used, because of its ability to alter cellular activity and function without cellular death.

[012] A targeting molecule can be attached to Cholera toxin subunit A through a chemical bond, or the composition can be prepared as a chimera using techniques of recombinant DNA. The conjugate can be used to specifically target cells having receptors to which the targeting molecule binds and inducing a cellular response in such cells. Accordingly, the invention provides a fusion protein comprising the amino acid sequence encoding a target molecule and Cholera toxin subunit A. This invention also provides a recombinant nucleic acid molecule comprising an isolated nucleic acid molecule encoding targeting molecule and cholera toxin subunit A. See U.S. Patent Nos. 4,666,837; 4,336,336; 5,906,820; 5,989,545; 6,043,057; 5,080,989; and 4,468,382; and U.S. Patent Publication

No. 2002/0006893. The teachings of all of these references are incorporated herein in their entirety to the extent not inconsistent with the teachings herein.

[013] In another embodiment, a CTA-TM conjugate comprises a CTA protein and a TM, wherein said protein or TM include a polypeptide at least 80%, or at least 85% identical, more preferably at least 90% or 95% identical, still more preferably at least 96%, 97%, 98% or 99% identical to CTA and at least one of the TMs described herein, respectively.

[014] By "% similarity" for two polypeptides, is intended a similarity score produced by comparing the amino acid sequences of the two polypeptides using the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711) and the default settings for determining similarity. Bestfit uses the local homology algorithm of Smith and Waterman (Advances in Applied Mathematics 2:482-489, 1981) to find the best segment of similarity between two sequences.

[015] By a protein having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a CTA protein is intended that the amino acid sequence of the protein is identical to the reference sequence except that the protein sequence may include a ratio of up to five amino acid alterations per each 100 amino acids of the reference amino acid of the CTA. In other words, to obtain a protein having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

[016] As a practical matter, whether any particular polypeptide or protein is at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to CTA or TMs, or fragments thereof, can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

[017] In a specific embodiment, the invention relates to engineering of a compound involving conjugating Substance P to a cholera toxin (SP-CTA) that suppresses pain cells while

minimizing cell death. The term "Substance P" as used herein refers to Substance P and suitable analog's thereof. See for example, U.S. Patent Publication 2004/0253248.

[018] Cholera toxin binds specifically to the surface receptors, GM-1 glanglioside, of neurons. Cholera toxin is typically used to uncouple G-protein and increase cyclic Adenosine Monophosphate concentration within cells[5].

[019] In a another embodiment, the subject invention pertains to a method for reducing pain in a subject comprising administering to the subject a therapeutically effective amount of the conjugate SP-CTA.

[020] In a specific embodiment, the subject invention pertains to a method of treating a NK-1R-associated disorder in a subject, which comprises administering to the subject an amount of the pharmaceutical composition comprising a therapeutically effective amount of the conjugate comprising SP-CTA and a pharmaceutically acceptable carrier thereby treating the disorder associated with the NK-1R. These disorders or diseases include but are not limited to: respiratory conditions (e.g. asthma, allergic rhinitis), ophthalmic conditions (e.g. conjunctivitis), cutaneous conditions (e.g. allergic dermatitis, dermatitis by contact, psoriasis), intestinal conditions (e.g. ulcerative colitis, Crohn's disease), gastrointestinal tract, central nervous system disorders such as anxiety and psychosis, inflammatory diseases such as rheumatoid arthritis and inflammatory bowel diseases, as well as pain in any of the aforesaid conditions, including migraine.

[021] Other disorders or diseases include but are not limited to: Alzheimer's disease, multiple sclerosis, attenuation of morphine withdrawal, cardiovascular changes, oedema, such as oedema caused by thermal injury, chronic inflammatory diseases such as rheumatoid arthritis, asthma/bronchial hyperactivity and other respiratory diseases including allergic rhinitis, inflammatory diseases of the gut including ulcerative colitis and Crohn's disease, ocular injury and ocular inflammatory diseases, proliferative vitreoretinopathy, irritable bowel syndrome and disorders of bladder function including cystitis and bladder detrusor hyperreflexia, demyelinating diseases such as multiple sclerosis and amyotrophic lateral sclerosis, asthmatic disease, small cell carcinomas, in particular small cell lung cancer, depression, dysthymic disorders, chronic obstructive airways disease, hypersensitivity disorders such as poison ivy, vasospastic diseases such as angina and Reynauldis disease, fibrosing, and collagen diseases such as scleroderma and eosinophilic fascioliasis, reflex sympathetic dystrophy such as shoulder/hand syndrome, addiction disorders such as alcoholism, stress related somatic disorders, neuropathy, neuralgia, disorder related to immune enhancement or suppression such as systemic lupus erythmatosis conjunctivitis, vernal conjunctivitis, contact dermatitis, atopic dermatitis, urticaria, and other eczematoid dermatitis and emesis; central nervous system disorders such as anxiety, depression, psychosis and schizophrenia; neurodegenerative disorders such as AIDS related dementia, senile dementia of the Alzheimer type, Alzheimer's disease and Down's syndrome; demyelinating diseases such as multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease) and other neuropathological disorders

such as peripheral neuropathy inflammatory diseases such as inflammatory bowel disease, irritable bowel syndrome, psoriasis, fibrositis, ocular inflammation, osteoarthritis and rheumatoid arthritis, allergies such as eczema and rhinitis; hypersensitivity disorders such as poison ivy; ophthalmic diseases such as conjunctivitis, vernal conjunctivitis, dry eye syndrome, and the like; cutaneous diseases such as contact dermatitis, atopic dermatitis, urticaria, and other eczematoid dermatitis; oedema; such as oedema caused by thermal injury; addition disorders such as alcoholism; stress related somatic disorders; reflex sympathetic dystrophy such as shoulder/hand syndrome; dysthymic disorders; neuropathy, such as diabetic or peripheral neuropathy and chemotherapy-induced neuropathy; postherpetic and other neuralgias; asthma; osteoarthritis; rheumatoid arthritis; and especially migraine.

[022] In addition to Substance P, CTA is conjugated with other targeting molecules that assist in targeting CTA to g-protein receptors. Examples of such targeting molecules include, but are not limited to, endorphins, and particularly in the case of an analgesic, beta-endorphin (Fries, DS (2002), Opioid Analgesics, In Williams DA, Lemke TL, *Foye's Principles of Medicinal Chemistry* (5 ed.), Philadelphia: Lippincott Williams & Wilkins), enkephalins (particularly Met enkephalin and Leu-enkephalin, [Met]-enkephalin is Tyr-Gly-Gly-Phe-Met and[Leu]-enkephalin has Leu in place of Met), endomorphins (e.g., endomorphin-1; Tyr-Pro-Trp-Phe-NH2, and endomorphin-2; Tyr-Pro-Phe-Phe-NH2) and the dynorphins. Other targeting molecules specific to G-protein receptors is provided in Br J Pharmacol 144: S4-S62 (2005). Other targeting molecules that are contemplated for conjugation to CTA include, but are not limited to, free compound serotonin, bradykinin, bombesin, calcitonin, cholecystokinin, neurotensin, glucagon, secretin, somatostatin, motilin, vasopressin, oxytocin, prolactin, thyrotropin, an angiotensin, galanin, neuropeptide Y, thyrotropin-releasing hormone, gonadotropnin-releasing hormone, growth hormone-releasing hormone, luteinizing hormone, glucosylamine, lactylamine, leucine, glutamate and amino cholines.

[023] The term "covalently coupled" as used herein is intended to mean that the coupled moieties are directly bonded to each other, or indirectly bonded to each other, such as by a linker.

[024] The term "linkage" as used herein is intended to mean a bond or group formed by chemical reaction between two moieties such that the moieties are covalently coupled. Methods for the preparation of a linkage such as an amide bond are described in Houben-Weyl, Methoden der organischen Chemie (Methods of Organic Chemistry), Volume 15/2; Bodanszky et al., in "Peptide Synthesis", E. Gross & J. Meienhofer (Eds), Academic Press, Y. Wiley, New York, 1976. Further reactions are detailed in R. C. Larock, Comprehensive Organic Transformations: A Guide to Functional Group Preparations, Wiley-VCH, 2nd ed., 1999.

[025] The subjects to be treated or whose tissue may be used herein may be a mammal, or more specifically a human, horse, pig, rabbit, dog, cat, monkey, or rodent. In the preferred embodiment the subject is a human

[026] The invention includes the pharmaceutically acceptable salts and complexes of all the compounds described herein. The salts include but are not limited to the following acids and bases. Examples of suitable inorganic acids include, but are not limited to: hydrochloric acid, hydrofluoric acid, hydrobromic acid, hydroiodic acid, sulfuric acid and boric acid. Examples of suitable organic acids include but are not limited to: acetic acid, trifluoroacetic acid, formic acid, oxalic acid, malonic acid, succinic acid, tartaric acid, maleic acid, fumaric acid, methanesulfonic acid, trifluoromethanesulfonic acid, benzoic acid, glycolic acid, lactic acid, citric acid and mandelic acid. Examples of suitable inorganic bases include, but are not limited to: ammonia, hydroxyethylamine and hydrazine. Examples of suitable organic bases include, but are not limited to, methylamine, ethylamine, trimethylamine, triethylamine, ethylenediamine, hydroxyethylamine, morpholine, piperazine and guanidine. The invention further provides for the hydrates and polymorphs of all of the compounds described herein.

[027] In one embodiment, the pharmaceutical carrier may be a liquid and the pharmaceutical composition would be in the form of a solution. In another equally preferred embodiment, the pharmaceutically acceptable carrier is a solid and the pharmaceutical composition is in the form of a powder or tablet. In a further embodiment, the pharmaceutical carrier is a gel and the pharmaceutical composition is in the form of a suppository or cream. In a further embodiment, the compound may be formulated as part of a pharmaceutically acceptable transdermal patch.

[028] A solid carrier can include one or more substances which may also act as flavoring agents, lubricants, solubilizers, suspending agents, fillers, glidants, compression aids, binders or table-disintegrating agents, it can also be an encapsulating material. In powders, the carrier is a finely divided solid that is in admixture with the finely divided active ingredient. In tablets, the active-ingredient is mixed with a carrier having the necessary compression properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain up to 99% of the active ingredient. Suitable solid carriers include, for example, calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, polyvinylpyrrolidine, low melting waxes and ion exchange resins.

[029] Liquid carriers are used in preparing solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The active ingredient can be dissolved or suspended in a pharmaceutically acceptable liquid carrier such as water, an organic solvent, a mixture of both, or pharmaceutically acceptable oils or fats. The liquid carrier can contain other suitable pharmaceutical additives such as solubilizers, emulsifiers, buffers, preservatives, sweeteners, flavoring agents, suspending agents, thickening agents, colors, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid carriers for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric and polyhydric alcohols, e.g. glycols) and their derivatives, and oils (e.g. fractionated coconut oil and arachis oil) For parenteral administration, the carrier can

also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid carriers are useful in sterile liquid form compositions for parenteral administration. The liquid carrier for pressurized compositions can be halogenated hydrocarbon or other pharmaceutically acceptable propellant, which are useful for intranasal administration.

[030] Liquid pharmaceutical compositions which are sterile solutions or suspensions can be utilized for intramuscular, intrathecal, intratracheal, epidural, intraperitoneal or subcutaneous injections. Sterile solutions can also be administered intravenously. The compounds may be prepared as a sterile solid composition that may be dissolved or suspended at the time of administration using sterile water, saline, or other appropriate sterile injectable medium. Carriers are intended to include necessary and inert binders, suspending agents, lubricants, flavorants, sweeteners, preservatives, dyes and coatings.

[031] In one embodiment the pharmaceutical composition further comprises a cytokine. Examples of cytokines include but are not limited: transforming growth factor beta, epidermal growth factor family, fibroblast growth factors, hepatocyte growth factor, insulin-like growth factors, B-nerve growth factor, platelet-derived growth factor, vascular endothelial growth factor, interleukin 1, IL-1 receptor antagonist, interleukin 2, interleukin 3, interleukin 4, interleukin 5, interleukin 6, IL-6 soluble receptor, interleukin 7, interleukin 8, interleukin 9, interleukin 10, interleukin 11, interleukin 12, interleukin 13, angiogenin, chemokines, colony stimulating factors, granulocyte-macrophage colony stimulating factors, erythropoietin, interferon, interferon gamma, leukemia inhibitory factor, oncostatin M, pleiotrophin, secretory leukocyte protease inhibitor, stem cell factor, tumor necrosis factors, and soluble TNF receptors.

[032] The compound can be administered in the form of a sterile solution or suspension containing other solutes or suspending agents, for example, enough saline or glucose to make the solution isotonic, bile salts, acacia, gelatin, sorbitan monoleate, polysorbate 80 (oleate esters of sorbitol and its anhydrides copolymerized with ethylene oxide) and the like.

[033] Examples of suitable pharmaceutical carriers include any of the standard pharmaceutically accepted carriers known to those of ordinary skill in the art. Examples of such pharmaceutical carriers include, but are not limited to, phosphate buffered saline solution, water, emulsions such as oil/water emulsions or a triglyceride emulsion, various types of wetting agents, tablets, coated tablets and capsules. A suitable pharmaceutically acceptable carrier may be selected taking into account the chosen mode of administration.

[034] Besides containing an effective amount of the compounds described herein the pharmaceutical compositions may also include suitable diluents, preservatives, solubilizers, emulsifiers, adjuvant and/or carriers.

[035] The resulting pharmaceutical compositions may be liquids or lyophilized or otherwise dried formulations. Examples of suitable diluents include, but are not limited to, Tris-HCL, Tris-

acetate and Tris-phosphate. The diluents employed may vary in their buffer contents pH and/or ionic strength. Examples of representative additives that may be used in the present invention include, but are not limited to: albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Plurpnic F68, bile acid salts), solubilizing agents (e.g., Thimerosal, benzyl alcohol), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the protein, complexation with metal ions, or incorporation of the material into or onto particulate preparation of polymeric compounds such as polylactic acid, polyglycolic acid, polyvinyl pyrrolidone, etc. or into liposomes, microemulsions, micelles, unilamellar or multimeller vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance of the compounds.

[036] Examples of optional ingredients which may be included in the pharmaceutical compositions of the present invention include antioxidants, e.g., ascorbic acid; low molecular weight (less than about the residues) polypeptides, i.e., polyarginine or tripeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; amino acids, such as glycine, glutamine acid, aspartic acid, or arginine; chelating agents such as EDTA; and sugar alcohols such as mannitol or sorbitol.

[037] The choice of composition will depend on the physical and chemical properties of the compounds. Controlled or sustained release compositions include formulation of lipophilic deposits (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines) and compounds coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms of protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

[038] Optimal dosages to be administered may be determined by those skilled in the art, and will vary with the particular compound in use, the strength of the preparation, the mode of administration, and the advancement of the disease condition. Additional factors depending on the particular subject being treated, including subject age, weight, gender, diet and time of administration, will result in a need to adjust dosages. Administration of the compound may be effected continuously or intermittently. in any treatment regimen, the composition may be administered to a patient either singly or in a cocktail containing two or more targeted toxins, other therapeutic agents, compositions, or the like, including, but not limited to, immunosuppressive agents, tolerance-inducing agents, potentiators and side-effect relieving agents. Particularly preferred are immunosuppressive agents useful in suppressing allergic reactions of a host. Preferred immunosuppressive agents include prednisone, prednisolone, DECADRON (Merck, Sharp & Dohme, West Point, Pa.), cyclophosphamide, cyclosporine, 6-mercaptopurine, methotrexate, azathioprine and i.v. gamma globulin or their combination. Preferred potentiators include monensin. ammonium chloride.

perhexiline, verapamil, amantadine, and chloroquine. All of is these agents are administered in generally-accepted efficacious dose ranges such as those disclosed in the Physician's Desk Reference, 41st Ed., Publisher Edward R. Barnhart, N.J. (1987).

[039] In the treatment, an appropriate dosage level will generally be about 0.001 to 50 mg per kg patient body weight per day that can be administered in single or multiple doses. Preferably, the dosage level will be about 0.005 to about 25 mg/kg, per day; more preferably about 0.01 to about 10 mg/kg per day; and even more preferably about 0.05 to about 1 mg/kg per day.

Example 1

(a) Materials and Methods

Animals

[040] Male Sprague Dawley rats (200 – 300 g) were housed in pairs and supplied standard rat chow and water *ad libitum* in the University of Florida's vivarium, which is an AAALAC certified facility.

[041] Intrathecal injections were performed under isoflurane anesthesia via lumbar puncture between L4 and L5. All animal procedures in this project were reviewed and approved by the University of Florida's Institutional Animal Care and Use Committee.

Synthesis of SP-CTA

[042] CTA was purchased from List Biological Laboratories inc. (Cambell, CA). CTA has two cysteine residues in the C-terminal region 15,27 therefore these cysteine residues were used to attach substance P to CTA. The synthesis of SP-CTA was accomplished using a modification of Pierce Biotechnology inc.'s maleimide protein cross-linking procedure. The synthesis was carried out in two stages. The first stage was to link maleimide to the N-terminus of substance P by combining a 5 fold excess of Sulfosuccinimidyl 4-N-maleimidomethyl cyclohexane-1-carboxylate (Sulfo-SMCC) with substance P in phosphate buffered saline (PBS, pH 7.4). The mixture was incubated at room temperature for 1 hour. The substance P maleimide conjugate was separated from unreacted Sulfo-SMCC using a Sephadex G-10 (30 X 1.5 cm) column eluted with PBS. For the second phase of the synthesis the substance P maleimide conjugate was linked to the two cysteines on CTA by adding a 10 fold excess of the conjugate to CTA in PBS. This mixture was then incubated at room temperature for another hour. The SP-CTA was then separated from the unreacted substance P maleimide conjugate, washed with PBS three times and concentrated using Centricon Plus-20 filters. A sample of the final product was evaluated by western blots. Briefly, the sample was run on 4-20% PAGE gels, transferred to PVDF membranes and then probed with antibodies to either substance P or the catalytic subunit of cholera toxin. A secondary antibody coupled to horse radish peroxidase and

enhanced chemiluminescence were used to visualize the bands. Figure 1 illustrates the synthetic pathway as well as western blots of the final product.

Cell Culture

[043] Chinese Hamster Ovary cells stably expressing NK1 receptors (CHO-NK1) (a generous gift from Dr. James Krause, Neurogen Corp.22) were plated on 100 mm plates for cAMP assays or 13 mm cover slips in 24 well culture plates for immunocytochemistry experiments. The cultures were grown in F12K media, 10% Fetal Bovine Serum, 1% L-glutamine, 1% penicillin-Streptomyosin, 25mM Hepes buffer, and G418 (500ìg/ml). The cells were cultured at 37oC in a 5% CO2 atmosphere.

cAMP Assay

[044] To assay cAMP levels in the cell cultures Sigma inc's (St. Louis, MO) Direct cAMP Enzyme Immunoassay was used according to the manufacturer's instructions. Briefly, the media on the cell cultures was removed and the cells were washed once with PBS (pH 7.4). The PBS was removed and 1 ml 0.1 M HCL was added to the cells. The cells were scraped from the plates into the HCL solution, sonicated and centrifuged (600 g, 10 minutes, 5^{O}C). The protein in each sample was measured using Bio-Rad's (Hercules, CA) protein assay. The cAMP was acetylated with the addition of 100 µl of the kit's acetic anhydride solution. A 100 µl sample was then neutralized with 50 µl of the kit's neutralizing buffer and the samples were added to the kit's 96 well plates that were pre-absorbed with antibodies to cAMP. A standard curve and controls were set up as suggested by the manufacturer. A cAMP- alkaline phosphatase conjugate (50 µl) was added to the wells and the solution was incubated for 2 hours. The plates were then washed 3 times and 200 µl of p-nitrophenyl phosphate solution (substrate) was added to each well and the plates were incubated for 1 hour. The reaction was stopped with 50 µl 0.1 M HCL and the plate was read at 405 nm. The concentration of cAMP in the samples was extrapolated from the data collected for the cAMP standards and expressed as the number of moles of cAMP per mg protein.

Immunocytochemistry

[045] Rats were euthanized with pentobarbital and immediately transcardially perfused with ice cold PBS and then ice cold 4% paraformaldehyde in phosphate buffered saline (PBS)(pH 7.4). Cell cultures on cover slips were washed with PBS and fixed with 4% paraformaldehyde in PBS. The spinal cords were removed and post fixed overnight in 4% paraformaldehyde in PBS. The tissue was cryoprotected in 30% sucrose, mounted and sectioned in a cryostat (-20oC)(10-20 μm) and mounted on slides. The sections or cells were then blocked with 3% normal goat serum for 60 minutes with 0.75% triton X-100. The primary antibody was then added to the blocking solution (1:500-5,000) and the sections were incubated for 48 hours at 4oC. The sections or cells were washed (8 X 5 mins) in PBS. Following the wash the sections or cells were incubated for 1 hour at room temperature in PBS with a secondary antibody that was coupled to horse radish peroxidase. The sections were washed (8 X 5 mins) and then treated with diaminobenzidine. The cultured cells were washed similarly and labeled using rhodamine labeled tyramide as described by the manufacturer (NEN, Boston, MA).

Thermal Nociception Assay

[046] Thermal nociception was measured using the method of Hargreaves et al.[9]. Briefly, the rats were placed on a clear glass surface and allowed 15 minutes to accommodate to the enclosure. An infrared light was directed onto a hind paw's plantar surface approximately in the middle of the foot. The latency for the animal to remove its foot from the path of the light was used as the dependent measure for thermal sensitivity.

Statistics

[047] Data were analyzed using a one way ANOVA followed by Dunnett's post-hoc test, one way repeated measures ANOVA, or paired ttests as appropriate. Significance was assigned to $p < 0.05$.

Results

Synthesis of SP-CTA

[048] The neuropeptide substance P was coupled to the catalytic subunit of cholera toxin (CTA) using the bifunctional linking agent sulfosuccinimidyl 4-N-maleimidomethyl cyclohexane-1-carboxylate (Sulfo-SMCC) as indicated in figure 1A. Briefly, the Sulfo-SMCC was reacted with the N-terminal amine of substance P to form an amide linkage to the maleimide group. The substance P – maleimide was then conjugated to CTA through two cysteine residues in the C-terminal region of the CTA protein. The final product was washed and concentrated by centrifugation in Centricon filters with a cutoff of 5kd. The success of the synthesis was confirmed on western blots by using antibodies

to both substance P and CTA. As demonstrated in figure 1B the final product produced bands on the western blot with a molecular weight of approximately 30kd that reacted with antibodies to substance P and CTA indicating a successful coupling of substance P to CTA (SP-CTA).

In Situ Evaluation of SP-CTA

[049] SP-CTA was tested on Chinese Hamster Ovary cells that were stably transfected with the NK1 receptor 22 (CHO-NK1). To verify selective uptake of SP-CTA by the cells, the cells were incubated over night in either SP-CTA (1 μg/ml) or CTA (1 μg/ml) alone. The CHO-NK1 cells were then fixed and prepared for immunocytochemistry with antibodies to CTA using a rhodamine-tyramide amplification system. As illustrated in figure 2A only the SP-CTA treated cells demonstrated an uptake of CTA indicating that linkage of CTA to substance P was required for the conjugate to be internalized.

[050] We further evaluated the functionality of the SP-CTA by examining the ability of the conjugate to stimulate cAMP production in CHO-NK1 cells. Figure 2B demonstrates the concentration response relationship for SP-CTA when the SP-CTA is applied for 1 hour and the cAMP was measured after culturing the cells for an additional 48 hours. The time course of SP-CTA's effect on cAMP production was evaluated by treating the cells for 1 hour with 100 ng/ml SP-CTA and then harvesting the cells for cAMP analysis 1, 2, 3 and 4 days following exposure to the conjugate. As demonstrated in figure 2C, SP-CTA's effects on cAMP peaked at 1 day in the CHO-NK1 cells and remained significantly elevated for 3 days.

[051] To further verify the selectivity of SP-CTA two additional experiments were performed. Figure 2D demonstrates that 48 hours following a 4 hour exposure to 100 ng/ml of either substance P, CTA or SP-CTA only SP-CTA treated CHO-NK1 cells produced an increase in cAMP production. When 100 ng/ml of substance P, CTA and SP-CTA remained in the culture media for the full 48 hours the substance P treated cells and the SP-CTA treated cells had significantly elevated levels of cAMP, whereas the CTA treated cells did not differ from control cells (Figure 2E). These findings indicate that substance P activation of NK1 receptors can stimulate adenylate cyclase, but that the effects of SP-CTA on cAMP production outlast any stimulation of the NK1 receptors produce by the substance P portion of the conjugate.

In Vivo Evaluation of SP-CTA

[052] Previous work with lethal toxins coupled to substance P demonstrated that neurons expressing NK1 receptors are necessary for the expression of thermal hyperalgesia. Therefore, the inventors believe that uptake of SP-CTA by NK1 receptor expressing neurons in the spinal cord

would stimulate the cells and produce thermal hyperalgesia. To test this idea rats were injected intrathecally with SP-CTA, CTA or saline (20 μl) via lumbar puncture under isoflurane anesthesia. In an initial group of rats (N = 6 per treatment group) 50 ìg of SP-CTA or CTA was injected. Twenty four hours following the injection of SP-CTA, but not CTA, the animals were agitated and aggressive toward their cage mates. These animals were euthanized by pentobarbital overdose and prepared for immunocytochemistry of the spinal cords to evaluate the phosphorylation of cAMP response element-binding protein (CREB). We chose to examine CREB because elevated levels of cAMP lead to the phosphorylation of this transcription factor 23. We found that SP-CTA treatment resulted in a large increase in the phosphorylation of CREB in the spinal cord dorsal horn (Figure 3), while the injection of CTA produced levels of phosphorylated CREB that were similar to saline injected animals.

[053] A dose response relationship and time course was determined for SP-CTA using the hind paw thermal nociception assay of Hargreaves et al.[9]. Figure 4A demonstrates that intrathecally administered SP-CTA (N = 10 rats per dose) has a biphasic dose response relationship 24 hours following the injections with peak thermal hyperalgesia observed at a dose of approximately 1 μg. At 10 μg of SP-CTA the animals demonstrated agitated behaviors similar to the first group that received 50 μg; therefore, no higher doses were tested. Finally, to determine the time course of action of SP-CTA 1 μg was injected intrathecally (N = 20 rats) and thermal nociception was tested before injections and 1 to 4 days following the injection. As illustrated in figure 4B the peak of thermal hyperalgesia occurred 1 day following the injection. Recovery occurred over the next three days; however, a complete return to baseline paw withdrawal thresholds was not observed.

(c) Discussion and Conclusion

[054] These experiments demonstrated that SP-CTA can be internalized and that it can elevate cAMP activity. When the A subunit of cholera toxin was conjugated to substance P the conjugate selectively acted on NK-1 expressing cells. Once inside the cholera toxin stimulated the ribosylation of the cell's G protein. This ribosylation stimulated the enzyme adenylate cyclase, which increased the production of cyclic Adenosine Monophosphate (cAMP).

[055] These results demonstrate another option and different usage for target toxins. As stated in the introduction section, in the past targeted toxins, like substance P-Saporin, have been used to kill the cells responsible for hyperalgesia. The work done in this experiment with SP-CTA used a different option; Cellular manipulation instead of death. This shows that specific cells can be targeted with a modified target toxin and the function of the cell can be customized. These results provide another potential option for pain management.

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[056] While various embodiments of the present invention have been shown and described herein, it will be obvious that such embodiments are provided by way of example only. Numerous variations, changes and substitutions may be made without departing from the invention herein. The teachings of all patents and other references cited herein are incorporated herein by reference to the extent they are not inconsistent with the teachings herein.

Claims

What is claimed is:

1. A conjugate comprising a cholera toxin subunit A covalently coupled to a targeting molecule, said targeting molecule having the ability to bind to g-protein coupled receptor.

2. The conjugate of claim 1, wherein said g-protein coupled receptor is expressed on a neural cell.

3. The conjugate of claim 1 wherein the targeting molecule is substance P, an endorphin, an enkephalin, a dynorphin, or an endomorphin.

4. The conjugate of claim 1, wherein the targeting molecule is substance P.

5. The conjugate of claim 1 wherein the cholera toxin subunit A and the targeting molecule are coupled together via a linker.

6. A pharmaceutical composition, comprising: a pharmaceutically acceptable carrier; and a therapeutically effective amount of the conjugate of claim 1.

7. The pharmaceutical composition of claim 6 wherein the targeting molecule is substance P, an endorphin, an enkephalin, a dynorphin, or an endomorphin.

8. A method of modulating neuron cellular activity in a subject, said method comprising administering to said subject an effective amount of a conjugate according to claim 1.

9. A method for reducing pain in a subject comprising administering to the subject a therapeutically effective amount of the conjugate according to claim 1.

10. The method of claim 8, wherein said targeting molecule is substance P.

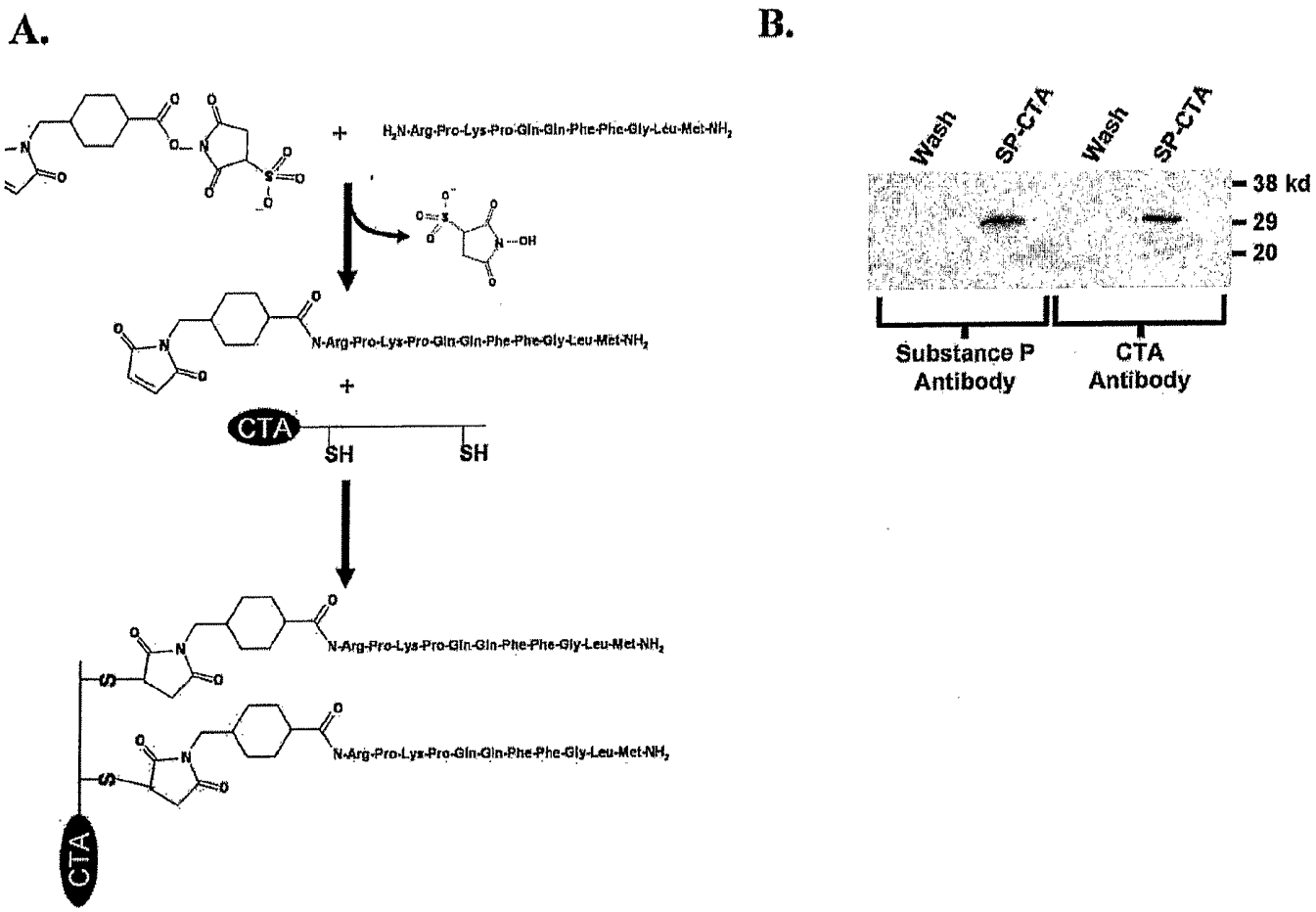

A.
B.
H2N-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2
N-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2
CTA
SH
SH
Wash
SP-CTA
Wash
SP-CTA
38 kd
29
20
Substance P Antibody
CTA Antibody

FIG 1

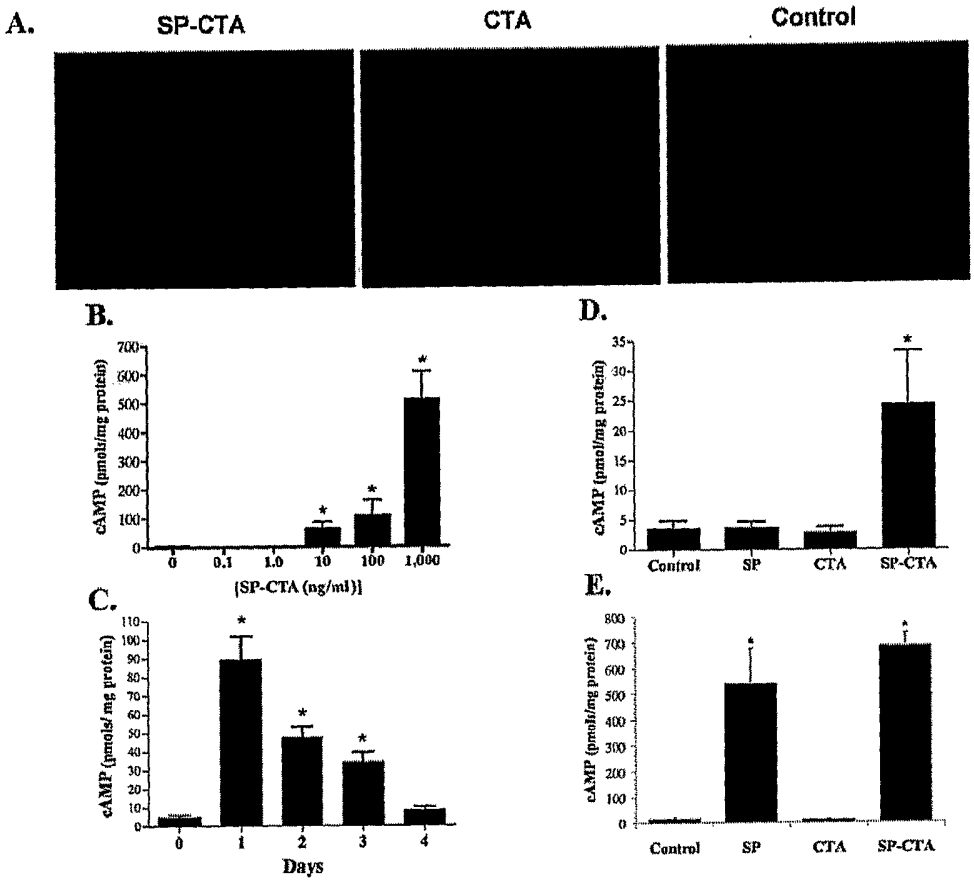

A.
SP-CTA
CTA
Control
B.
cAMP (pmols/mg protein)
[SP-CTA (ng/ml)]
C.
cAMP (pmols/ mg protein)
Days
D.
cAMP (pmol/mg protein)
Control
SP
CTA
SP-CTA
E.
cAMP (pmols/mg protein)
Control
SP
CTA
SP-CTA

FIG. 2

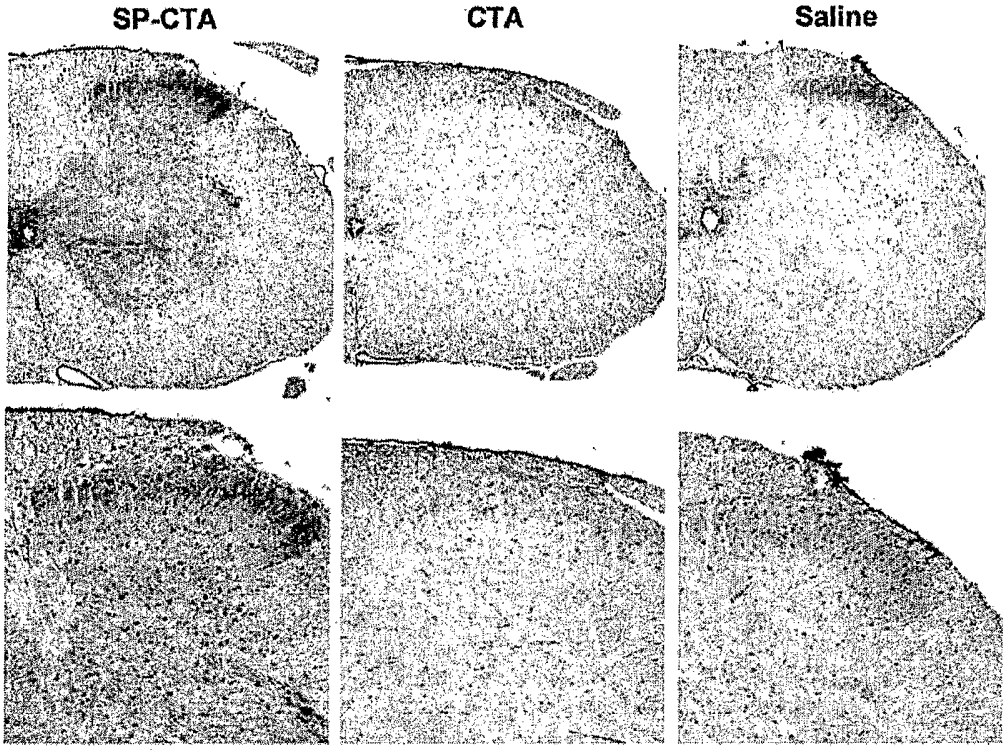


FIG. 3

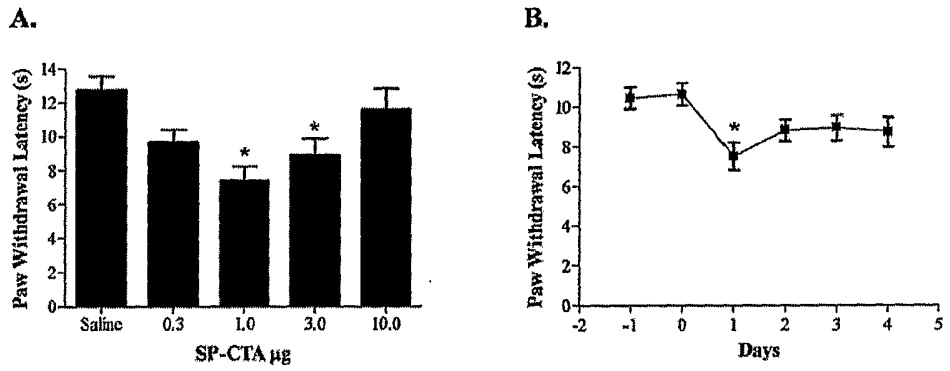

A.
Paw Withdrawal Latency (s)
14
12
10
8
6
4
2
0
Saline
0.3
1.0
3.0
10.0
SP-CTA µg
B.
Paw Withdrawal Latency (s)
12
10
8
6
4
2
0
-2
-1
0
1
2
3
4
5
Days

FIG. 4